

A<sup>3</sup> Sub B1  
cont.

23. (Amended) The assay platform according to claim 1 wherein the polymer matrix binds to polypeptide target molecules having a molecular weight up to 350 kDa in an amount of at least 2 µg/cm<sup>2</sup>.

26. (Amended) The assay platform according to claim 1 wherein the binding ligand binds to a polynucleotide target molecule.

27. (Amended) The assay platform according to claim 1 wherein the binding ligand binds to a mRNA target molecule.

28. (Amended) The assay platform according to claim 1 wherein the binding ligand binds to a DNA target molecule.

#### REMARKS

Claim 1 has been amended to recite that the polymer matrix --binds to-- target molecules --through a binding ligand--. Support for this amendment is found in the specification at, for example, pages 5-6, ¶0016 and original claim 1. See *In re Gardner*, 177 USPQ 396, 397 (CCPA 1973) and MPEP §§ 608.01(o) and (I). Claims 16-23 and 26-28 have been amended to conform with the amendments to claim 1 wherein "is capable of binding" has been replaced with --binds to--.

Claim 10 has been amended to add --Matrix Assisted Laser Desorption Ionization--, which is the acronym for "MALDI." Support for this amendment is found in the specification at, for example, page 4, ¶0013.

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### **Restriction Requirement**

As requested by the Examiner in the Office Action, election of the subject matter of Group I (i.e., claims 1-38) is affirmed. (See Paper No. 5 at 1-4).

### **§112 Rejections**

Claims 1, 16-23 and 26-28 have been rejected under 35 USC §112, second paragraph. (Paper No. 5 at 4). In making the rejection, the Examiner asserted that the term "capable of" is vague and indefinite because "[i]t is unclear if the polymer matrix or the binding ligand actually does what is claimed." (Id.).

Initially, we direct the Examiner's attention to pages 5-6, ¶ 0016 of the specification, wherein the phrase "polymer matrix" is defined to comprise "a plurality of polymer molecules wherein at least some of the polymer molecules have at least one binding ligand covalently attached thereto" and that "a binding ligand shall be understood to mean a moiety that binds to a target molecule ...." Thus, the polymer matrix does bind to, or is capable of binding to, a target molecule through the binding ligand. One skilled in the art when reading the claims, especially in light of the specification, would have been able to ascertain with a reasonable degree of precision and particularity the particular area set out and circumscribed by the claims. See e.g., *Ex parte Wu*, 10 USPQ2d 2031, 2032-33 (BPAI 1989). Nothing more is required.

With a view toward furthering prosecution, however, claim 1 has been amended to recite that "the polymer matrix binds to target molecules through a binding ligand." Claims 16-23 and 26-28 also have been amended to

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conform with the amendment to claim 1. In view of the foregoing, it is respectfully submitted that claims 16-23 and 26-28 are clear and that the rejection should be withdrawn.

Claim 10 was also rejected under §112, second paragraph. (Paper No. 5 at 4). In making this rejection, the Examiner asserted that "[t]he acronym 'MALDI' is not defined in the claim so that those who are ordinary skills in the art would know applicant intended meaning." (*Id.*).

With a view toward furthering prosecution, claim 10 has been amended to include the phrase "Matrix Assisted Laser Desorption Ionization" for which "MALDI" is an acronym. Accordingly, the rejection has been rendered moot and should be withdrawn.

For the reasons set forth above each rejection under §112, second paragraph has been rendered moot. Accordingly, withdrawal of each rejection, respectfully is requested.

#### **§102(b) Rejection**

Claims 1-9, 12-23 and 26-30 have been rejected under 35 USC §102(b) as anticipated by International Publication No. WO 92/03732 assigned to Bioprobe International, Inc. ("Bioprobe"). (Paper No. 5 at 5).

For the reasons set forth below, the rejection, respectfully, is traversed.

In making the rejection, the Examiner asserted that Bioprobe "teaches an assay platform that has a coating material ... for use in assay

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methods involving solid phase materials (pg. 3, lines 28-32; page 4, lines 8-29; pg. 6, lines 32-35 to pg. 7, line 19) ... [that the] coating comprise a polymer such as dextran (pg. 10, lines 7-21) that binds to the solid phase materials ... [that a] spacer is also included ... (pg. 7, lines 30-35 to pg. 8, lines 1-2; pg. 8, lines 25-35 to pg. 9, lines 1-26) ... [and that the] ligand is comprised of biologically active molecules to target molecules such as DNA and RNA (pg. 9, lines 27-35)."

The Examiner then concluded that "[s]ince the polymer matrix of the reference [Bioprobe] contains all of the features required by the instant assay platform, i.e., the polymer binds to the substrate, the polymers are crosslinked to other polymers and attached to a ligand, it is *inherent* that the density of the polymer of the reference would also be at least 2  $\mu\text{g}/\text{cm}^2$ ." [emphasis added].

(Id.).

Bioprobe discloses compositions and methods for preparation of assay systems, in particular, "solid phase" systems. (p. 1, ins. 7-8). In the assay system, Bioprobe discloses "water-soluble compounds (both monomers and polymers) including hydrophobic moieties that *bind tightly* to, e.g., the plastics commonly used as solid phases ...." (p. 3, ln. 33 - p. 4, ln. 1). These compounds "further carry reactive functional groups (e.g., hydrazide or 2-(N-methylpyridyl groups) which form *stable covalent bonds* with ligands ...." (p. 4, ins. 1-4). Bioprobe discloses a general formula for such reagents as:



wherein  $R^H$  represents a hydrophobic moiety which develops *substantial nonspecific interactions* with a hydrophobic material, such as solid phase

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materials .... (*Id.* at Ins. 8-15). Bioprobe further distinguishes between the type of binding between (1) the solid phase material and the coating material and (2) the coating material and the ligand.

The desired coating material is provided in a hybrid form as a combination of (1) one or more hydrophobic moieties designed to maximize and therefore stabilize the *nonspecific binding* of the coating material with the solid phase materials, and (2) reactive groups capable of forming *stable covalent bonds* with ligand of interest in a specific manner. (p. 7, Ins. 1-9).

This spacer serves the purpose of effectively establishing two domains in the molecule: a hydrophobic domain which *interacts with* the hydrophobic materials (primarily, through nonspecific binding mechanisms); and a reactive group-presenting domain, which permits the *binding* to specific site(s) of the ligand in readily accessible form. (*Id.* at Ins. 23-30).

Polymers that *bind tightly* to plastics and form *covalent bonds* with the nucleophilic groups of proteins (e.g., amino or SH groups) are also contemplated. (p. 11, Ins. 28-31).

Bioprobe further discloses methods for avoiding crosslinking of the polymeric materials. For example, Bioprobe discloses that "[w]hereas the direct reaction of a reactive dihydrazide (e.g. adipic dihydrazide) tends to result in the formation of insoluble products (probably due to crosslinking), the addition of small amounts of hydrophobic hydrazides to the dihydrazide reaction mixture

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tends to ***inhibit crosslinking***, permitting a longer reaction time and a more complete reaction." (p. 10, lns. 14-21).

In all of the Bioprobe examples, to prepare the surface of a solid phase for binding to ligands, the coating material was applied to the solid phase, optionally incubated for a period of time, and then washed off. (See e.g. p. 12, ln. 21 – p. 32, ln. 18). Table 2 shows resistance to detergent (*i.e.*, Tween-20) of two different coating materials (dextran-hydrazide and dextran-phenylhydrazide) applied to polystyrene microtiter plates. (p. 20, lns. 4-15). For both coating materials, at 10% Tween-20, 26.91% and 66.74% binding of rabbit anti-HRP was disclosed. (*Id.*).

As is well settled, anticipation requires "identity of invention." *Glaverbel Societe Anonyme v. Northlake Mktg. & Supply*, 33 USPQ2d 1496, 1498 (Fed. Cir. 1995). Each and every element recited in a claim must be found in a single prior art reference ***and arranged as in the claim***. [emphasis added]. *In re Marshall*, 198 USPQ 344, 346 (CCPA 1978); *Lindemann Maschinenfabrik GMBH v. American Holst and Derrick Co.*, 221 USPQ 481, 485 (Fed. Cir. 1984).

Initially, we note that the rejection is cobbled together by pulling disclosure from disparate parts of the Bioprobe specification. The table below demonstrates that the rejection on page 5 of the Office Action pieces together snippets of disclosure spanning pages 3-10 of the Bioprobe disclosure.

Location in Bioprobe
pg. 3, lines 28-32
pg. 4, lines 8-29

pg. 6, lines 32-35
pg. 7, line 19
pg. 10, lines 7-21
pg. 7, lines 30-35 to pg. 8, lines 1-2
page 8, lines 25-35
pg. 9, lines 1-26
pg. 9, lines 27-35

In short, the rejection relies on nine different sound bites from eight different pages in an attempt to reconstruct the presently claimed assay platforms. It is respectfully submitted that having to string together such disparate disclosures does not meet the strict requirement of being "arranged as in the claim." For this reason alone the rejection should be withdrawn.

The rejection also appears to substitute a lesser "obviousness" standard, *i.e.*, whether it would be obvious to look to the various snippets identified by the Examiner – to arrive at the claimed rejection. But this type of standard shifting is not appropriate in a §102(b) rejection, which requires not only that each and every claimed element be present in a prior art document but also that such elements be "arranged as in the claim." The rejection has not – and cannot – identify a single citation from Bioprobe containing each and every element recited in the rejected claims. Thus, it is respectfully submitted that the rejection is legally insufficient to support a rejection under §102(b).

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Assuming *arguendo* that the rejection is legally sufficient, which is not admitted, we demonstrate below that it is factually flawed and should be withdrawn for the following additional reasons.

The rejection admits that Bioprobe does not disclose a density of the polymer matrix on the substrate as recited in, e.g. claim 1 and that it must rely on inherency for that element. (Paper No. 5 at 5) ("it is inherent that the density of the polymer of the reference would also be at least  $2 \mu\text{g}/\text{cm}^2$ ").

As is well settled, an Examiner bears the initial burden of establishing a *prima facie* case to deny patentability. *In re Piasecki*, 223 USPQ 785, 788 (Fed. Cir. 1984). To support a rejection based on inherency, an examiner must provide factual and technical grounds establishing that the inherent feature necessarily flows from the teachings of the prior art. *Ex parte Levy*, 17 USPQ2d 1461, 1464 (PBAI 1990). It is not enough that the asserted inherent property may arise from the facts disclosed. *In re Oelrich*, 212 USPQ 323, 326 (CCPA 1981) (inherency must flow as a necessary conclusion from the prior art, not simply a possible one); and *Glaxo Inc. v. Novopharm Ltd.*, 34 USPQ2d 1565, 1567 (Fed. Cir. 1995) ("Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient").

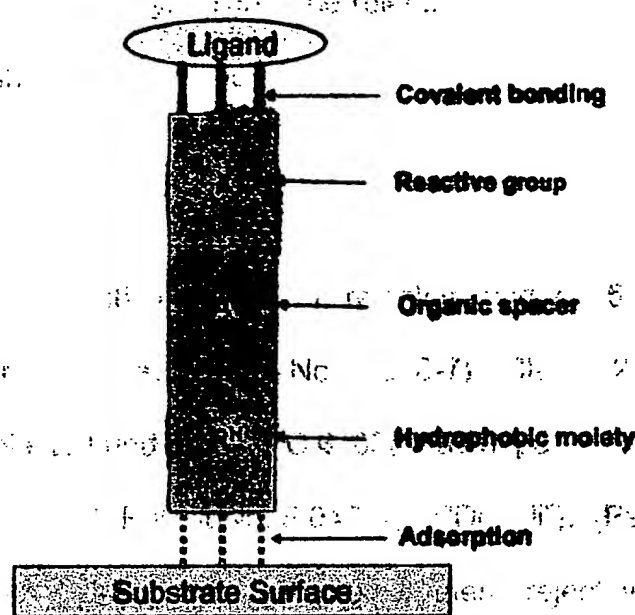
Here, the Examiner's sole basis for the inherency of the claimed "at least  $2 \mu\text{g}/\text{cm}^2$ " element in Bioprobe is the bald assertion that "the polymer binds to the substrate." Nothing more is provided.



It is respectfully submitted that the assertion "the polymer binds to the substrate," without more, does not meet the evidentiary burden of coming forth with "a basis in fact and/or technical reasoning" to support the inherency reasoning. *Ex parte Levy*, 17 USPQ at 1464. Simply because the "polymer binds to the substrate" does not provide a factual basis or shed any light on why *ipso facto*, the polymer must be present on the surface of the substrate at a density of "at least 2  $\mu\text{g}/\text{cm}^2$ ." Because the rejection fails to provide any facts or technical reasoning to support its bald assertion, the rejection should be withdrawn for this reason as well.

Notwithstanding the factual insufficiency of the rejection, we submit concurrently herewith a DECLARATION OF DR. WILLIAM KAPPEL UNDER 37 CFR §1.132 demonstrating that Bioprobe does not disclose inherently, or otherwise, a density of the polymer matrix on the substrate of at least 2  $\mu\text{g}/\text{cm}^2$  as recited in, e.g. claim 1. (See Decl., ¶¶8 and 18).

According to Dr. Kappel, Bioprobe discloses adsorbing a tripartite reagent to a substrate by hydrophobic (non-covalent) interactions through the  $R^H$  portion of the reagent. (Decl., ¶¶10-11). As a consequence of the adsorption of the  $R^H$  portion of the Bioprobe reagent to e.g., a plastic substrate, Dr. Kappel states that, based on well known principles of adsorption chemistry, the Bioprobe reagent, at best, forms a monolayer on such a surface. (Decl., ¶¶12-14). As Dr. Kappel states, the Bioprobe reagent may be depicted as follows:



(Decl. ¶11).

Dr. Kappel also demonstrates that in view of the adsorption of the Bioprobe reagent onto plastic surfaces, the density of the Bioprobe reagent on such a surface would be about 300 ng/cm<sup>2</sup>. (Decl. ¶15). Dr. Kappel also observes that the presently claimed minimal polymer matrix density, i.e., "at least about 2 µg/cm<sup>2</sup>," is at least **six-fold higher** than the maximal possible polymer density disclosed in Bioprobe. (Decl., ¶¶16-17).

Thus, Dr. Kappel concludes that Bioprobe does not inherently, or otherwise, disclose the "at least 2 µg/cm<sup>2</sup>" element recited by the present claims. Based on Dr. Kappel's consideration of Bioprobe and the Examiner's inherency argument, it is respectfully submitted that the Examiner's conclusion that "the density of the polymer of the [Bioprobe] reference would be at least 2 µg/cm<sup>2</sup>" is flawed. As Dr. Kappel demonstrates, a density of the polymer on the substrate of at least 2 µg/cm<sup>2</sup> does not "necessarily flow" from Bioprobe. In fact, it is neither a

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"possibility" nor a "probability." Thus, the rejection is factually deficient and must be withdrawn for this reason as well.

### **§103(a) Rejections**

Claims 1-38 have been rejected under 35 USC §103(a) as unpatentable over Bioprobe. (Paper No. 5 at 6-7). Claims 22, 24-25 and 33-34 also have been rejected under 35 USC §103(a) as unpatentable over Bioprobe in view of Döbeli et al., U.S. Patent No. 5,047,513 ("Döbeli"). (Paper No. 5 at 7).

For the reasons set forth below, these rejections, respectfully are traversed.

Bioprobe is summarized above. Döbeli disclose metal chelate resins used for the purification of, e.g. proteins. (Col. 1, Ins. 58-62). The metal chelates have the structure of:



(Col. 2, ln. 10).

In making the rejection with respect to claims 1-38, the Examiner relied on the same disclosures in Bioprobe identified in the §102(b) rejection. (Paper No. 5 at 6). The Examiner acknowledged, however, that Bioprobe differs from the presently claimed invention in that it fails to "include the features such as a glass substrate (Claim 11) and the ligand being a metal chelate (Claim 24)." (*Id.*). To fill the acknowledged gap, the Examiner asserted that the features of the dependent claims "are either well known alternatives or constitute obvious variations ...." (*Id.* at 6-7).

In making the rejection with respect to claims 22, 24-25 and 33-34, the Examiner relied on the same disclosures in Bioprobe identified in the §102(b) rejection. (Paper No. 5 at 7). The Examiner acknowledged, however, that Bioprobe differs from the presently claimed invention in that it fails to "specifically include a metal chelate as the ligand." (*Id.*) To fill the acknowledged gap, the Examiner relied on Döbeli as teaching "a metal chelate for chromatography purification of proteins ...". (*Id.*)

The Examiner then concluded that "[i]t would have been obvious to ... modify the assay platform of Bioprobe by including [the] metal chelate as taught by Döbeli ...." (*Id.*).

As noted above, the Examiner bears the initial burden of presenting a *prima facie* case of unpatentability. If the Examiner cannot meet this burden, the applicant is entitled to a patent. *In re Piasecki*, 223 USPQ at 788 and *In re Oetiker*, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992).

It is fundamental that a *prima facie* case of obviousness must be based on facts, "cold hard facts." *In re Freed*, 165 USPQ 570, 571-72 (CCPA 1970). When the rejection is not supported by facts, it cannot stand. *Ex parte Saceman*, 27 USPQ2d 1472, 1474 (BPAI. 1993).

Here, each of the §103 rejections is infirm, and must be withdrawn for at least the same reasons identified above with respect to the §102(b) rejection, namely that the Examiner's reliance on inherency to meet the "at least 2 µg/cm<sup>2</sup>" element of, *e.g.*, claim 1 is factually and legally insufficient. As Dr. Kappel explains in his Declaration, at best, Bioprobe discloses coating a surface

of a substrate with a reagent to a density of about 300 ng/cm<sup>2</sup>, which is, at a minimum, ~~six-fold~~ less than the amount recited in the present claims. (Decl. ¶¶ 16-17). Neither §103 rejection fills this gap in Bioprobe identified by Dr. Kappel. Thus, as a matter of fact and law, both §103 rejections must be withdrawn. *In re Robertson*, 49 USPQ2d 1949, 1951 (Fed. Cir. 1999) ("The lack of novelty' upon which the Board based its conclusion of obviousness, however, was its finding of anticipation. Our rejection of that finding eliminates the sole basis of the Board's obviousness determination, which therefore cannot stand.").

For completeness, we identify below additional bases that require both §103 rejections to be withdrawn:

As is well settled, suggestion or motivation to make a combination as claimed must also be based on factual evidence. The "case law makes clear that the best defense for guarding against a hindsight-based obviousness analysis is the rigorous application of the *requirement for a showing of a teaching or motivation to combine*" the references applied. *Ecologchem Inc. v. Southern California Edison*, 56 USPQ2d 1065, 1073 (Fed. Cir. 2000); and see *In re Dembiczak*, 50 USPQ2d 1614, 1617 (Fed. Cir. 1999) ("Combining prior art references *without evidence* of such a suggestion, teaching, or motivation simply takes the inventor's disclosure as a blueprint for piecing together *the prior art* to defeat patentability-the essence of hindsight.").

Even when a rejection is based on a single reference, such as the rejection of claims 1-38 over Bioprobe alone, "there must be a showing of a suggestion or motivation to modify the teachings of that reference." *In re Kotzab*,

55 USPQ2d at 1317. And there also **"must be evidence** that a skilled artisan, confronted with the same problems as the inventor and with no knowledge of the claimed invention, **would select** the elements from the cited prior art [reference] for combination in the manner claimed." *Ecologchem Inc. v. Southern California Edison*, 56 USPQ2d at 1076 (citing *In re Rouffet*, 47 USPQ2d 1453, 1456 (Fed. Cir. 1998)).

Here, neither Bioprobe alone nor Bioprobe in combination with Döbell suggest or provide a motivation to use a polymer density on a substrate of "at least 2  $\mu\text{g}/\text{cm}^2$ " as claimed. Rather, when considered as a whole, Bioprobe discloses anchoring the reagent to a substrate by adsorption. Using the adsorption mechanism as disclosed in Bioprobe, however, Dr. Kappel explains that achieving the "at least 2  $\mu\text{g}/\text{cm}^2$ " as claimed is not possible. And, the Examiner has provided no evidence or technical reasoning to explain why one would depart from the Bioprobe method for anchoring the reagent to a substrate surface. Nor does the Examiner explain with evidence or technical reasoning why the density of polymer on the substrate surface that is achieved, approximately 300  $\text{ng}/\text{cm}^2$ , needs to be modified, let alone modified to the specific density recited in the claim - "at least 2  $\mu\text{g}/\text{cm}^2$ ." For these reasons as well, the §103 rejections are deficient and should be withdrawn.

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In view of the foregoing, reconsideration, entry of the amendments, consideration of the **DECLARATION OF DR. WILLIAM KAPPEL UNDER 37 CFR §1.132** and allowance of the claims, respectfully is requested. If the Examiner has any questions regarding this paper, please contact the undersigned.

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to the Commissioner For Patents, Washington, D.C. 20231, on January 29, 2003.

  
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**"Marked Up" Amendments to Claims Pursuant to Rule 1.121(c)**

1. (Amended) An assay platform comprising a substrate and a polymer matrix attached to the substrate, wherein the polymer matrix binds to [is capable of binding] target molecules through a binding ligand, wherein the polymer matrix comprises a plurality of polymer molecules, wherein at least some of the polymer molecules are covalently attached directly to the substrate, wherein at least some of the polymer molecules are crosslinked to other polymer molecules, wherein at least some of the polymer molecules have at least one binding ligand covalently attached thereto, and wherein the density of the polymer matrix on the substrate is at least 2  $\mu\text{g}/\text{cm}^2$ .

10. (Amended) The assay platform according to claim 1 wherein the substrate is a Matrix Assisted Laser Desorption Ionization (MALDI) plate.

16. (Amended) The assay platform according to claim 1 wherein the polymer matrix binds to [is capable of binding] target molecules having a molecular weight of less than 3.5 kDa in an amount of at least 1 nanomole/ $\text{cm}^2$ .

17. (Amended) The assay platform according to claim 1 wherein the polymer matrix binds to [is capable of binding] target molecules having a



molecular weight of 3.5 kDa to 500 kDa in an amount of 0.5  $\mu\text{g}/\text{cm}^2$  to 20  $\mu\text{g}/\text{cm}^2$ .

18. (Amended) The assay platform according to claim 1 wherein the polymer matrix binds to [is capable of binding] target molecules having a molecular weight of 10 kDa to 500 kDa in an amount of 1  $\mu\text{g}/\text{cm}^2$  to 20  $\mu\text{g}/\text{cm}^2$ .

19. (Amended) The assay platform according to claim 1 wherein the polymer matrix binds to [is capable of binding] target molecules having a molecular weight of 10 kDa to 350 kDa in an amount of 2  $\mu\text{g}/\text{cm}^2$  to 20  $\mu\text{g}/\text{cm}^2$ .

20. (Amended) The assay platform according to claim 1 wherein the polymer matrix binds to [is capable of binding] target molecules having a molecular weight of 10 kDa to 350 kDa in an amount of 3  $\mu\text{g}/\text{cm}^2$  to 15  $\mu\text{g}/\text{cm}^2$ .

21. (Amended) The assay platform according to claim 1 wherein the polymer matrix binds to [is capable of binding] target molecules having a molecular weight of 10 kDa to 350 kDa in an amount of 4  $\mu\text{g}/\text{cm}^2$  to 10  $\mu\text{g}/\text{cm}^2$ .

22. (Amended) The assay platform according to claim 1 wherein the binding ligand binds to [is capable of binding] a polypeptide target molecule.

23. (Amended) The assay platform according to claim 1 wherein the polymer matrix binds to [is capable of binding] polypeptide target molecules having a molecular weight up to 350 kDa in an amount of at least 2  $\mu\text{g}/\text{cm}^2$ .

26. (Amended) The assay platform according to claim 1 wherein the binding ligand binds to [is capable of binding] a polynucleotide target molecule.

27. (Amended) The assay platform according to claim 1 wherein the binding ligand binds to a [is capable of binding] mRNA target molecule.

28. (Amended) The assay platform according to claim 1 wherein the binding ligand binds to [is capable of binding] a DNA target molecule.

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**Principles in Adsorption to Polystyrene****(Bulletin No. 6, Second Edition 1997)**

**When considering the binding capacity of adsorbant plastic surfaces for bio-macromolecules, one must distinguish between the total amount of molecules that can be bound to the surface and the amount that can be bound and still remain biologically active. Both quantities are very much dependent on the nature of the molecules and the character of the surface.**

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Adsorption Forces   Adsorbing Surfaces   Geometric Estimation   Experimental Estimation  
Discussion   Table 1   Graphs (Figs. 1 - 12)

**Adsorption Forces**

The adsorption of molecules to a polystyrene surface is due to intermolecular attraction forces (van der Waals forces), to be distinguished from "true" chemical bonds, i.e. covalent bonds (through electron share) and ionic bonds (through stoichiometric charges of opposite signs), see [Fig. 1](#).

Intermolecular attraction forces are based on intramolecular electric polarities of which two types can be distinguished: alternating polarities (AP) and stationary polarities (SP), i.e. dipoles.

AP arises when molecules approach each other, thereby creating disturbances in each other's electron clouds. This causes synchronously alternating polarities in the molecules, which may establish a bond between them, as illustrated in [Fig. 2](#).

AP mediated binding is a common substance property, which is obviously the stronger, the larger the molecules implied. This is demonstrated by the fact that melting and boiling points increase with number of carbon atoms in the non-polar hydrocarbon series. Indeed, it is due to this force that non-polar molecules at all aggregate into liquids and solids.

In addition to the AP attraction forces, molecules may possess SP (stationary polarity) through which they can bind to each other simply by bedding dipole against dipole, as illustrated in [Fig. 1 \(a\)](#).

Compared with SP, AP attraction decreases drastically with increasing distance between the molecules. Thus, AP attraction is inversely proportional to the seventh power of the distance, whereas SP attraction is inversely proportional to only the second power of the distance. Hence, the former has a much shorter range than the latter.

In general, van der Waals mediated bonds are about 100 times weaker than ionic and covalent bonds. However, among SP mediated bonds the hydrogen bond takes up an exceptional position because it is up to 10 times stronger than the others and because of its crucial importance for the properties of water and for the specific behaviors of bio-molecules.

Chemical groups, which can take part in hydrogen bonding, in particular -OH, =OH, -NH<sub>2</sub>, =NH,

=N, are called hydrophilic, as opposed to hydrophobic groups lacking this ability. Accordingly, hydrogen bonds may be called hydrophilic bonds, as opposed to AP mediated bonds, which are called hydrophobic bonds. The AP mediated attraction is also called hydrophobic interaction.

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## Adsorbing Surfaces

The Nunc-Immuno® program includes two different types of adsorbant polystyrene surfaces, the POLYSORP® and the MAXISORP® surface. Whereas PolySorp predominantly presents hydrophobic groups, MaxiSorp has in addition many hydrophilic groups, which results in a fine patchwork of hydrophobic and hydrophilic binding sites.

In aqueous medium, a repelling effect exists between the PolySorp surface and hydrophilic macromolecules (i.e. rich in hydrophilic groups), because these molecules will rather tend to intermingle with the water molecules (i.e. be dissolved) by the strong hydrogen bonds than bind to the surface by the weak hydrophobic bonds.

On a MaxiSorp surface, however, adsorption of hydrophilic macromolecules will be greatly facilitated, because not only can this surface compete with the water molecules for binding the macromolecules by hydrogen bonds, but the molecules can also be captured from a much longer distance by the long-range hydrogen bond forces for establishment of both hydrogen bonds and eventually hydrophobic bonds (see [Fig. 3](#)).

On the other hand, hydrophobic macromolecules (i.e. deficient in or lacking hydrophilic groups) can only be loosely adsorbed to MaxiSorp, because this surface tends to bind water molecules by hydrogen bonds, against which the macromolecules cannot compete and therefore exhibit poor ability for displacing water molecules and hydrophobic adsorption free from water pocket interruptions.

On a PolySorp surface, however, no hindrance exists for stable hydrophobic adsorption of hydrophobic molecules, except that they may not be applicable in purely aqueous medium, wherefore addition of or substitution with detergent or organic solvents (e.g. ethanol or hexane) may be needed.

In summary, when no attention is paid to maintenance of specific activities, hydrophobic compounds bind preferably to PolySorp, and hydrophilic compounds preferably to MaxiSorp (see [Table 1](#)). However, with MaxiSorp binding events are more likely to occur, which means that adequate incubation conditions are more easy to establish—a fact that may extend the MaxiSorp application range well into the theoretical PolySorp domain. Nevertheless, considering the maintenance of the specific activities of the molecules (e.g. enzymatic, immunologic), which of course is crucial, the specific sites may well be obscured, impaired or destroyed through the binding to the surface.

Therefore, when constructing a solid phase assay, it is generally recommended to try adsorption to MaxiSorp first. If this does not work satisfactorily, it may be due to molecular malfunction, and one should then try PolySorp whereby specific activities may be maintained because of the different binding mechanism to this surface.

However with PolySorp, where molecules must come very close to the surface to establish hydrophobic bonds, one must anticipate more demanding incubation conditions, such as higher reactant concentration, longer duration, higher temperature, (more) agitation, to obtain adsorption efficiency comparable with MaxiSorp.

As mentioned above, van der Waals mediated bonds are relatively weak, wherefore they may be insufficient for stable bonding when they are few in number, i.e. when the molecules are small. For binding of small molecules, strong chemical bonds are needed. Ionic bonds would not do, because they normally dissociate in aqueous solution, leaving covalent bonds as the only possibility for direct, stable binding of small molecules. However, this difficulty may be overcome by using small

molecules linked to (indifferent) carrier macromolecules. Small molecules would in this context be e.g. peptides of less than 10 amino acids (corresponding to about 1500 dalton).

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## Geometric Estimation

Before making any experimental estimates of binding capacities on solid phase surfaces, it is worth making an estimate from geometric considerations of how many molecules can maximally be packed in one layer on a surface.

Taking immunoglobulin G antibody (IgG) as an example, and assuming that it is globular and packed in the densest monolayer packing (Fig. 4), the amount  $Q_{\text{GLOBE}}$  per  $\text{cm}^2$  will be:

$$Q_{\text{GLOBE}} = 2/\sqrt{3} \cdot MW/N \cdot 1/(2r)^2 \cdot 10^9 \text{ ng/cm}^2 = 300 \text{ ng/cm}^2$$

where:

MW = molecular weight of IgG =  $153,000 \text{ g} \cdot \text{mole}^{-1}$

N = Avogadro's number =  $6 \cdot 10^{23} \text{ mole}^{-1}$

$$r = \text{Stokes radius of IgG} = \frac{R \cdot T_{20}}{6 \cdot \pi \cdot \eta_{20} \cdot D_{20} \cdot N} \quad \text{cm}$$

R = gas constant =  $8.3 \cdot 10^7 \text{ g} \cdot \text{cm}^2 \cdot \text{sec}^{-2} \cdot \text{°K}^{-1} \cdot \text{mole}^{-1}$

$T_{20}$  = room temperature ( $20^\circ\text{C}$ ) =  $293^\circ\text{K}$

$\eta_{20}$  = viscosity of water at  $20^\circ\text{C}$  =  $1 \cdot 10^{-2} \text{ g} \cdot \text{cm}^{-1} \cdot \text{sec}^{-1}$

$D_{20}$  = diff. coeff. of IgG ref. to water at  $20^\circ\text{C}$  =  $4 \cdot 10^{-7} \text{ cm}^2 \cdot \text{sec}^{-1}$

However, according to various sources, the IgG molecule is rather a lens-shaped spheroid with a diameter, d, of about 15 nm and a thickness, t, of about 3 nm as illustrated in Fig. 5.

Assuming the densest packing of these spheroids in "upright" or "lying" position (Fig. 6), the respective  $Q_{\text{LENS}}$  values will accordingly be:

Upright position:

$$Q_{\text{LENS}} = 2/\sqrt{3} \cdot MW/N \cdot 10^9 \cdot 1/t^2 = 650 \text{ ng/cm}^2$$

Lying position:

$$Q_{\text{LENS}} = 2/\sqrt{3} \cdot MW/N \cdot 10^9 \cdot 1/d^2 = 130 \text{ ng/cm}^2$$

So, for geometrical reasons alone, the maximum amount of monolayer IgG that can be bound on a surface is  $650 \text{ ng/cm}^2$ . If an average is taken between the two  $Q_{\text{LENS}}$  figures, the final estimate would be  $400 \text{ ng/cm}^2$ .

Assuming that molecular weight is proportional with volume, Q will not change considerably within wide molecular weight limits, other things being equal, because of the low power relationship between volume and profile area of a body. Fig. 7 illustrates the relationship between Q and molecular weight for globular molecules.

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## Experimental Estimation

Let us, as an example, stay with IgG, a glycoprotein with a structure shown schematically in [Fig. 8](#).

On a MaxiSorp surface, one would expect an orientated adsorption in favor of exposing the antigen-recognizing sites, because this surface would favor a binding through the hydrophilic carbohydrate moiety associated with the non-recognizing leg of the molecule.

On a PolySorp surface, on the other hand, one would expect an adsorption in favor of obscuring the antigen-recognizing sites, because of the repelling effect between this surface and the carbohydrate moiety.

To investigate the actual adsorption conditions, the following experiment was designed (see [Fig. 9](#)):

PolySorp and MaxiSorp MicroWell® surfaces were coated with a dilution series of specific antibodies, starting with a concentration C well above saturation concentration, or with a corresponding dilution series mixed with unspecific antibodies to a constant total of C. The relative amount of specific antibody adsorbed in each case was determined by a sandwich ELISA for the antigen in question using excess antigen and excess HRP-conjugated specific antibodies.

When it is assumed that equal signals means equal amounts of specific antibody adsorbed in both dilution series, the quantity B/C is the fraction of the saturation concentration S formed by the maximum signal concentration A,

$$\text{i.e. } A = S \cdot B/C, \text{ or } S = A/B \cdot C$$

In [Fig. 10](#) are shown the results from experiments, designed as above, with four antibodies of different specificities. From the seemingly constant curve distances for MaxiSorp (MS) and PolySorp (PS) respectively, it is concluded that the adsorptions are independent of antibody specificity, and that they amount to the following quantities:

$$Q_{MS} = A_{MS}/B_{MS} \cdot C \cdot V/F \cdot 10^3 = 650 \text{ ng/cm}^2$$

$$Q_{PS} = A_{PS}/B_{PS} \cdot C \cdot V/F \cdot 10^3 = 220 \text{ ng/cm}^2$$

where:

$$A_{MS}/B_{MS} = 1/20$$

$$A_{PS}/B_{PS} = 1/60$$

$$C = \text{max. IgG conc.} = 100 \text{ } \mu\text{g/ml}$$

$$V = \text{reactant volume} = 0.2 \text{ ml}$$

$$F = \text{surface area} = 1.54 \text{ cm}^2$$

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## Discussion

Whereas  $Q_{MS}$  is identical with the geometric maximum estimate for upright molecules,  $Q_{PS}$  is only one third hereof, which can be explained by assuming that on PolySorp upright and lying molecules are present in equal numbers, as illustrated in [Fig. 11](#).

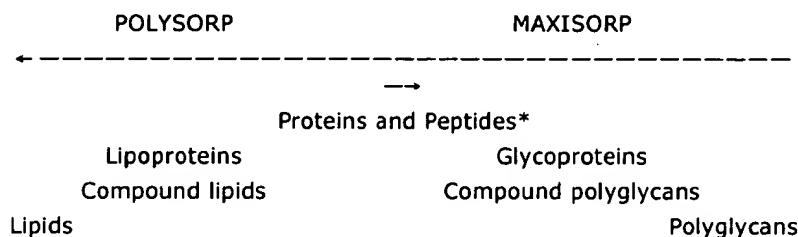
Because each IgG antibody can maximally bind two antigen molecules, this PolySorp decrease in number of adsorbed antibodies would have the greater effect, the smaller the antigen molecules are compared with the antibodies, as illustrated in [Fig. 12](#). This could, partly at least, explain the very low PolySorp signals for AFP, which has a molecular weight of less than half the weight of IgG, whereas the other antigens have 3 to 5 times the weight of IgG.

In addition, the variation of PolySorp to MaxiSorp maximum signal ratios may be due to differently obstructed affinities through the antibody adsorption to PolySorp, and/or due to different antibody-antigen affinities from one system to the other. In the ferritin system, the affinity seems relatively high since there is a long delay before maximum signal decline on MaxiSorp, which may be consistent with the high PolySorp maximum signal, i.e. the higher the affinity, the less it is obstructed by antibody adsorption to PolySorp.

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**Table 1**

Theoretical PolySorp and MaxiSorp preferences for adsorption of various bio-macromolecules.

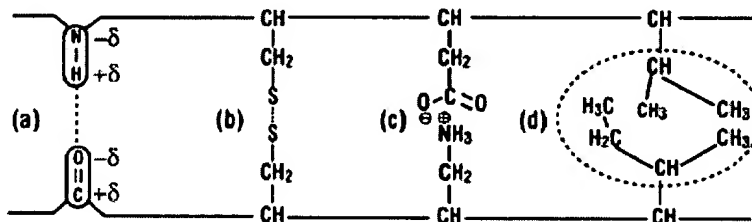


\*Surface preference is dependent on predominance of hydrophobic or hydrophilic amino acid residues in the molecules.

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## Graphs

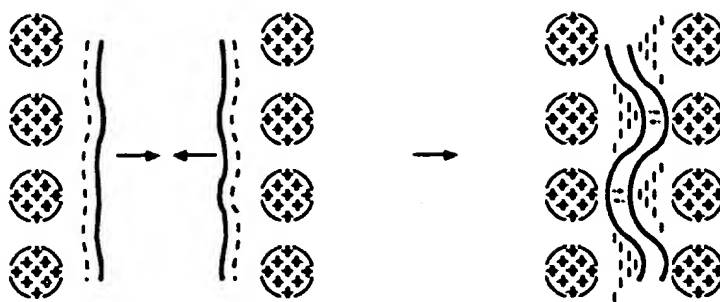
**Fig. 1**



The four main types of possible bonds between macromolecules. "True" chemical bonds are represented by a covalent disulphide bond (b) and an ionic bond between a carboxyl ion and an amino ion (c). Van der Waals mediated bonds are represented by a hydrogen bond between two dipoles (a) and an alternating polarity bond between hydrocarbon residues protruding from the macromolecules' backbones (d), where the encircled area indicates a water-deprived zone.

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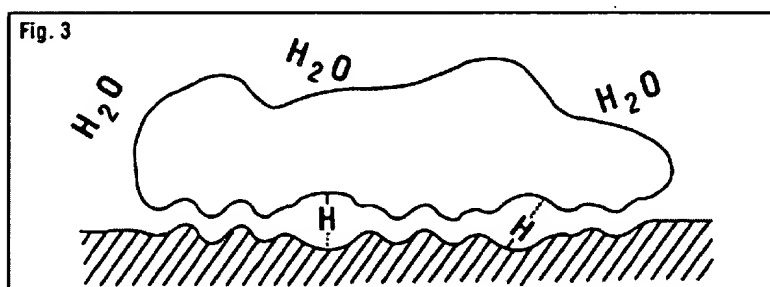
**Fig. 2**



Schematic illustration of how synchronously alternating polarities (AP), created by reciprocal electron cloud disturbances in approaching molecules, can establish a bond between the molecules. Transient, minus-charged electron cloud condensations in one molecule will attract reciprocally exposed, plus charged nuclear regions in the other molecule.

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**Fig. 3**

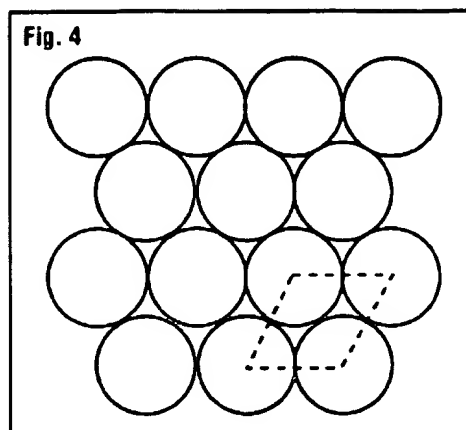


Schematic illustration of how a hydrophilic macromolecule can be firmly adsorbed to MaxiSorp by "squeezing" out the water between the molecule and the surface through the combined action of hydrogen bond and AP bound forces.

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**Fig. 4**

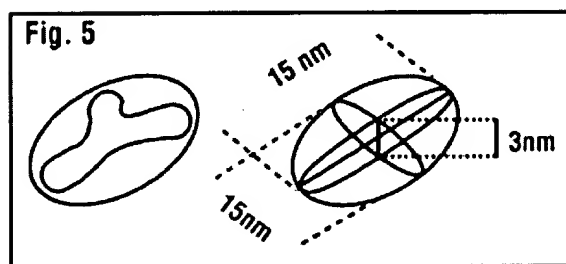




**The densest monolayer packing of globular molecules seen from above. The factor  $2/\sqrt{3}$  in the text formulas for surface binding capacities originates in this non-quadratic pattern.**

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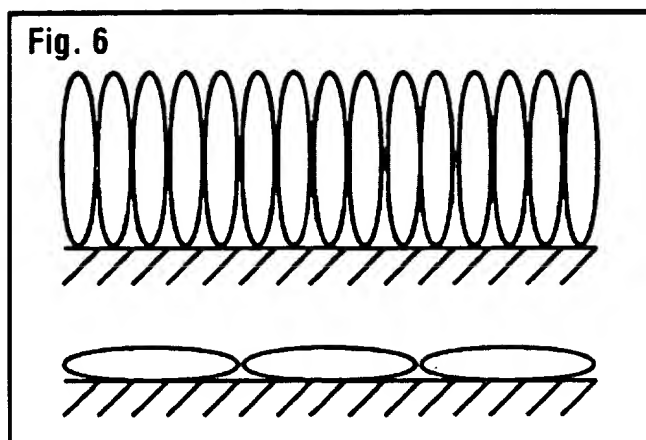
**Fig. 5**



**The Y-shaped IgG antibody will approximately take up the volume of a lens-shaped spheroid with a diameter of 15 nm and a thickness of 3 nm.**

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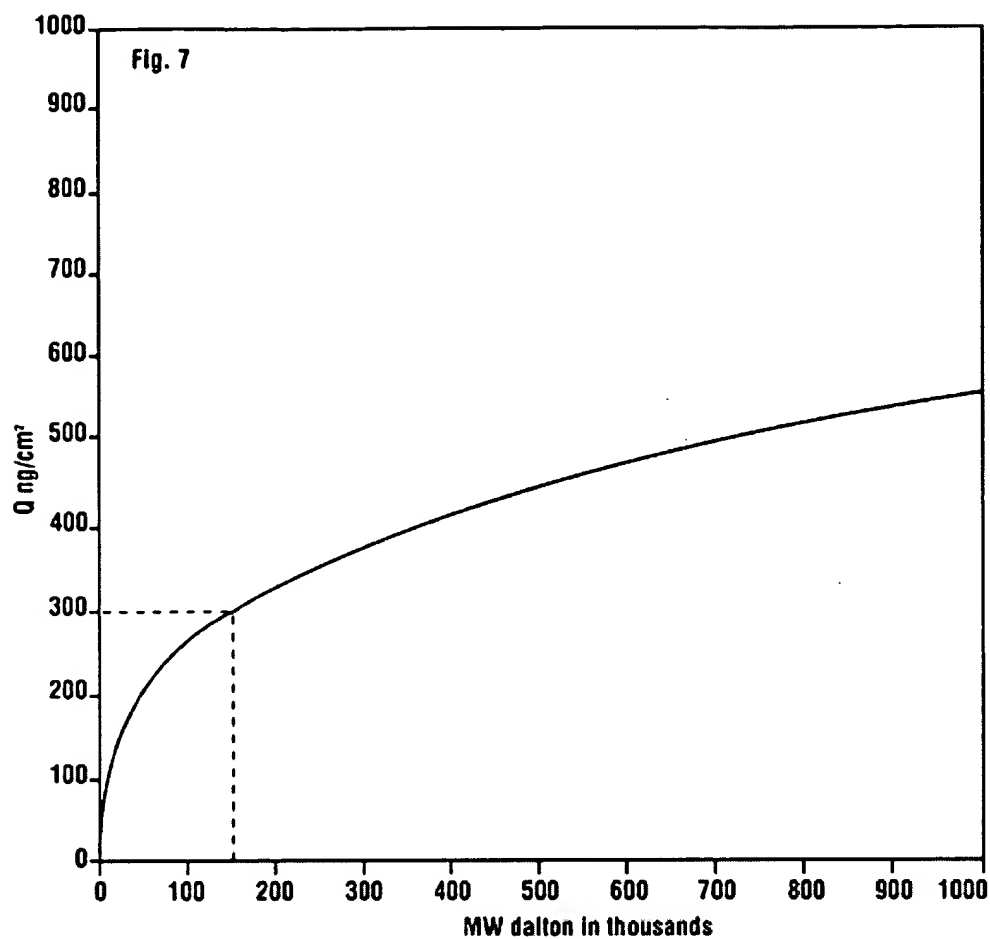
**Fig. 6**



**Profiles of the densest IgG packings on a surface illustrating the density ratio of 5 to 1 between molecules packed in upright position (above) and in lying position (below).**

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**Fig. 7**

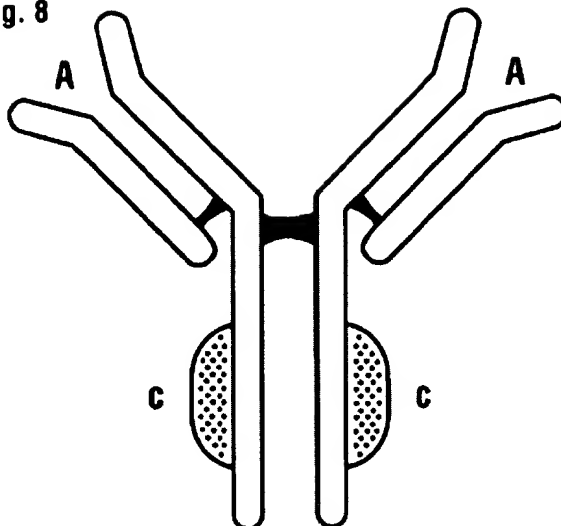


Relationship between monolayer weight density (Q) and molecular weight (MW) of globular molecules illustrating that within a 10-factor MW range Q will roughly vary within only a 2-factor range. The curve is extrapolated on the basis of an idealized IgG molecule with an MW of 153,000 (dashed lines).

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**Fig. 8**

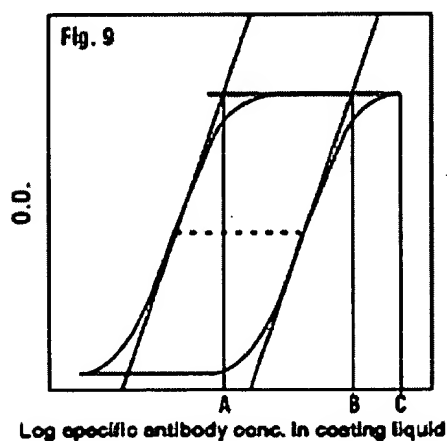
Fig. 8



Schematic illustration of the IgG antibody structure. Note the carbohydrate moiety (at C) associated with the leg opposite the antigen binding sites (at A) of the molecule.

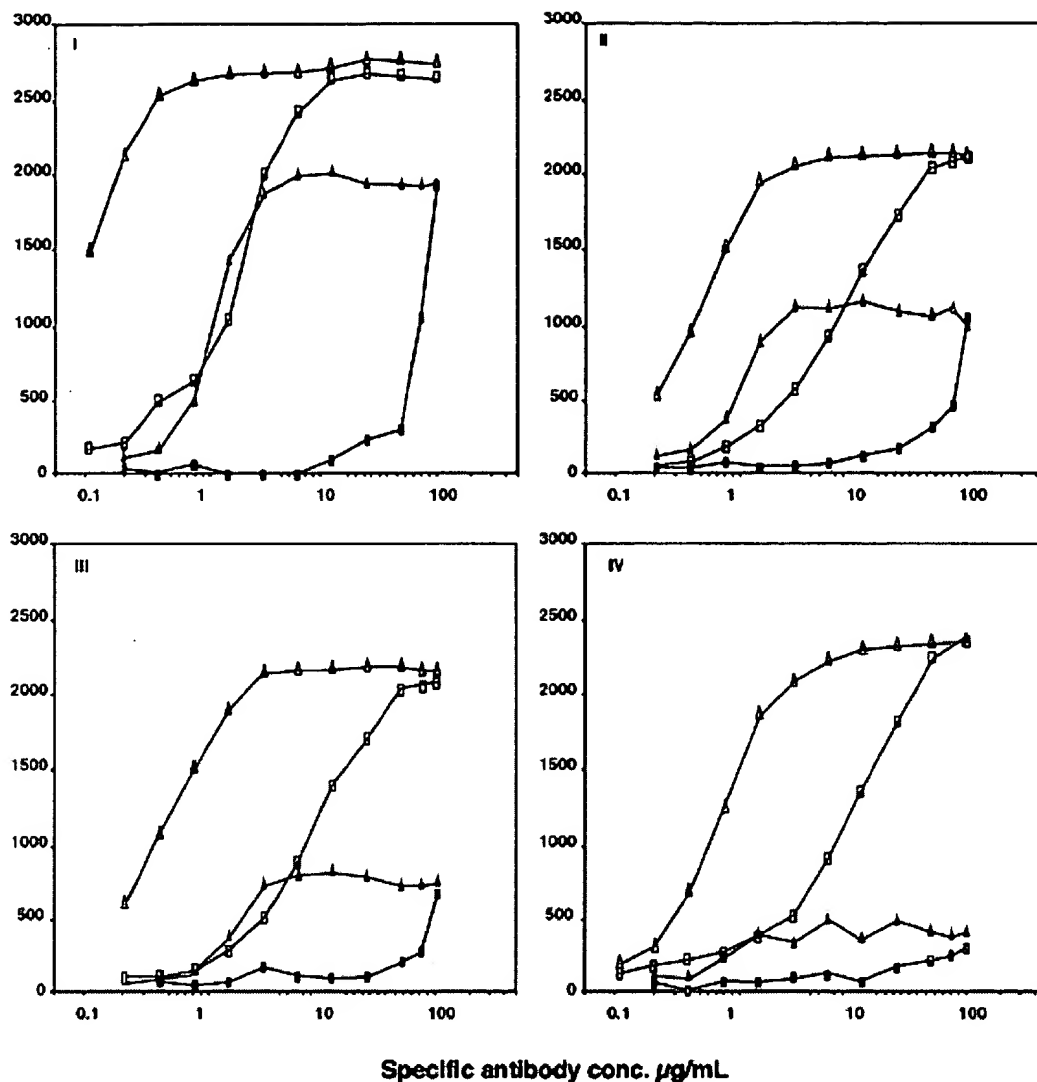
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Fig. 9



Expected results from ELISA experiments with a dilution series of first layer specific IgG-antibodies, starting with a concentration C well above surface saturation concentration S (left sigmoid curve), or with a corresponding dilution series mixed with unspecific IgG to a constant total of C (right sigmoid curve). The ratio between S and C is A/B, which is represented by the distance between the two curves (dashed lines).

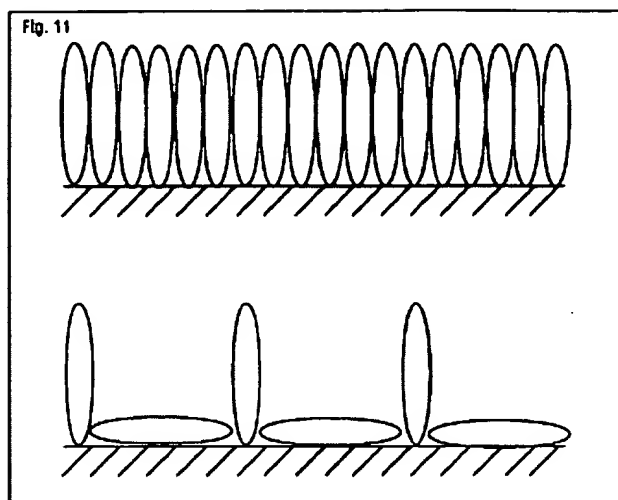
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**Fig. 10**

Results from experiments prospected in Fig. 9 with four different antibody/antigen systems on MaxiSorp (open symbols) and on PolySorp (filled symbols). I: ferritin antigen (MW 440,000); II: fibronectin antigen (MW 450,000); III: thyroglobulin antigen (MW 670,000); IV: AFP (a - foeto-protein) antigen. Note the seemingly constant curve distances for MaxiSorp and PolySorp respectively, regardless of the system in question. See text and Fig. 9 for further explanation.

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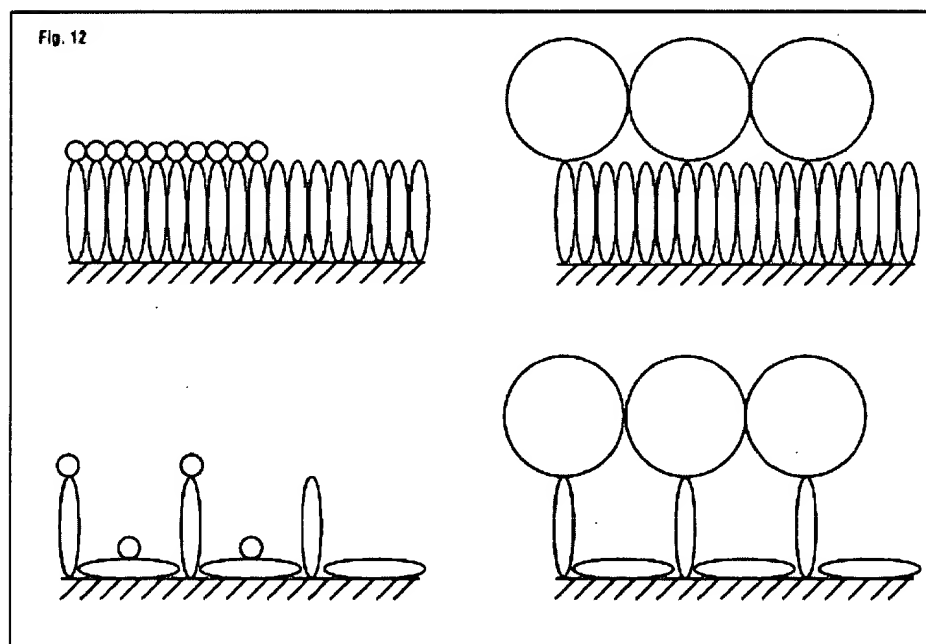
**Fig. 11**



**Profiles of IgG adsorption patterns on MaxiSorp (above) and PolySorp (below) which can explain the experimentally found ratio of 3 to 1 between the densities on the respective surfaces.**

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**Fig. 12**



**Profiles of second layer (antigen) binding to antibody-coated MaxiSorp (above) and PolySorp (below) surfaces illustrating how the presumptive difference between the IgG adsorption patterns may imply a PolySorp decrease in bound amounts of small antigen molecules (left), but not of large antigen molecules (right). It should be noted that the third layer consisting of HRP-conjugated antibodies would hardly influence the detection of this phenomenon, as HRP is a relatively small molecule (MW 40,000).**

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## **Related Links**

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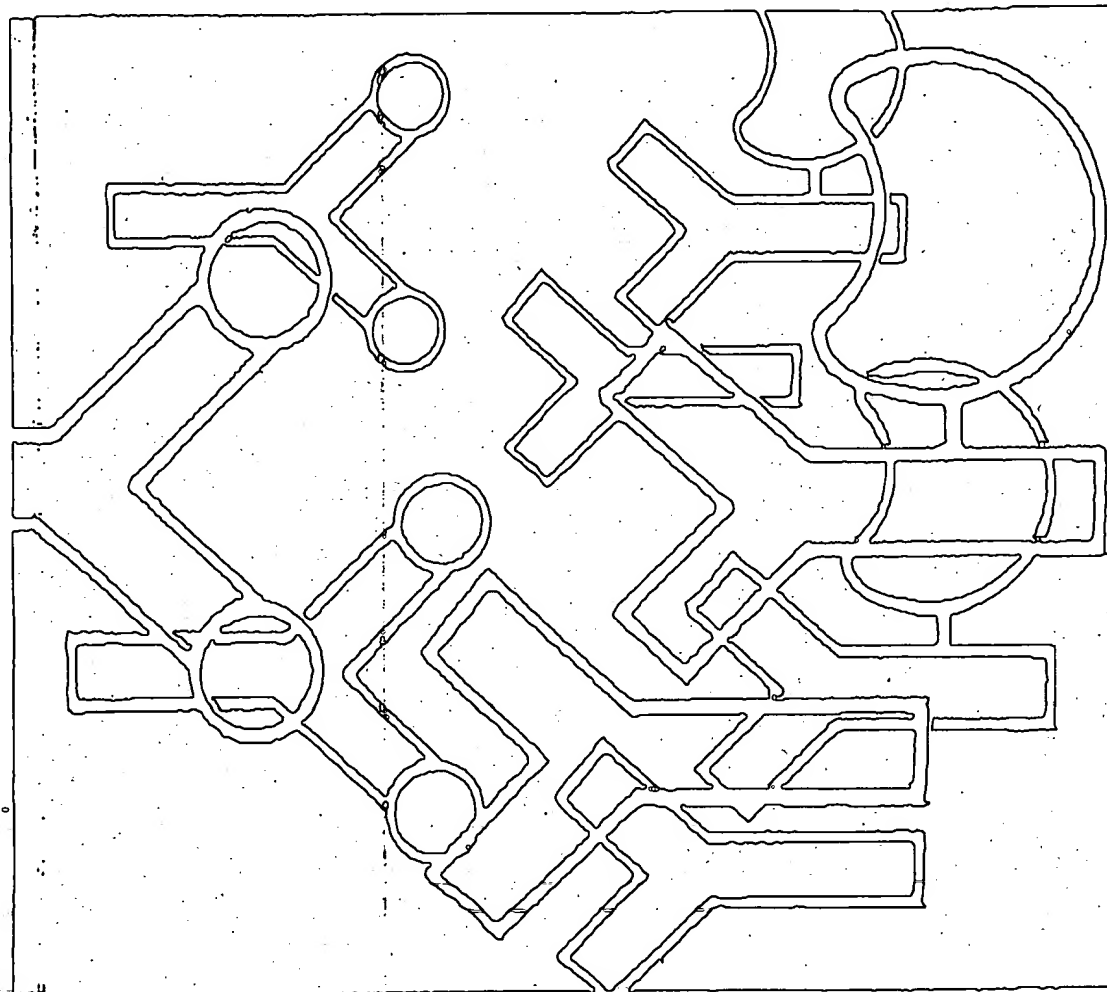
[Aspects of Nunc Maxisorp Microwell Certification \(Bulletin No.4, Second Edition 1997\)](#)

[Negative Edge Effect in Microwell® Elisa](#)

[Stability of Nunc-Immuno® Maxisorp® Surfaces](#)

[Nunc 384-Well Plate Design and Performance Evaluation](#)

ENZYME  
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FROM CONCEPT TO  
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S.S. DISHPANDI



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## 7

## Separation and Solid-Phase Systems

### SEPARATION SYSTEMS

#### Introduction

To quantitate the amount of analyte present in an enzyme immunoassay (EIA), the extent of reaction of the enzyme-labeled ligand (or antibody) with antibody (or the ligand) must be determined. Since the reaction between the ligand and its specific antibody does not produce a precipitate, it is necessary to effect a physical separation of the two forms before either or both forms can be quantitated. By definition, in all heterogeneous EIAs, a physical separation of the free and antibody-bound fractions is required. The homogeneous EIAs, in contrast, are separation-free systems; they do not require separation of the free and bound forms.

Separation techniques have become the critical issue in the development and advancement of immunoassays. Numerous techniques have been developed to separate labeled antigen-antibody complexes from the unbound labeled fraction. Removal of one of these labeled fractions enables the development of quite sensitive and relatively precise immunoassays. Regardless of methodology, either competitive or immunometric, or of the label, radioisotopic, enzymic, or fluorometric, the more efficient the separation of the bound from the unbound fraction, the more the sensitivity of the assay will be enhanced. In exchange for the sensitivity and specificity benefits obtained, these separation steps can be slow, difficult to automate, and may require multiple operations. This in turn increases the technical requirements for performing the assay.

Of the new assays or assay systems described during the past two decades, only about 10-20% require no separation step (Figure 7.1). Therefore, most immuno-

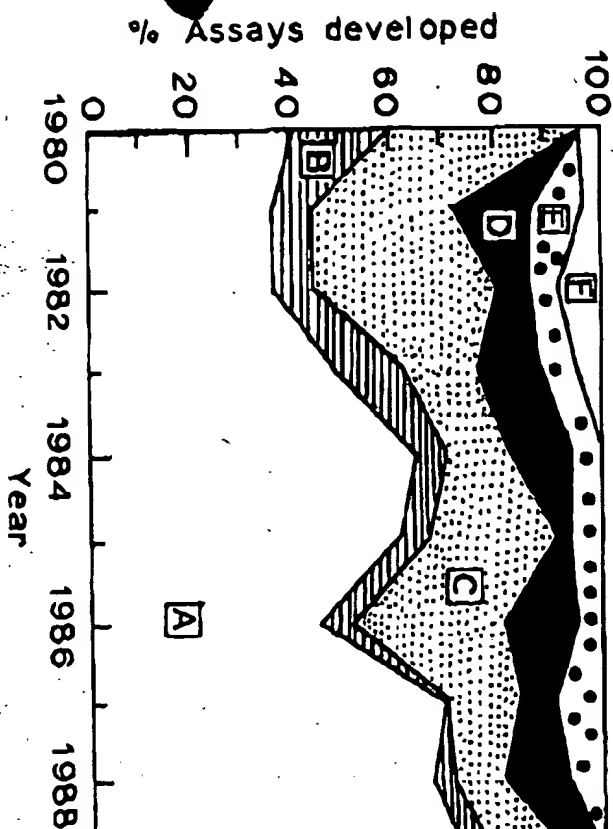


Figure 7.1. Trends over the past decade with respect to the use of different separation methods or no separation step (homogeneous, turbidimetric assays, etc.) in new immunoassays. The survey includes new immunoassays described in *Clinical Chemistry* from January 1980 to December 1989, and does not include articles on immunoassays concerned with immunoassay evaluation, comparison, improvement, automation, problems of interference, etc. The average sample size was 42 (range, 27 to 1980 to 57 in 1985). The adsorption methods used charcoal (a total of 28 assays over the decade) or Florisil® (one assay). The precipitation methods largely involved second antibody (32 assays) but also polyethylene glycol alone (31 assays), protein A (60 assays), and ammonium sulfate (two assays).

A: Solid-phase methods  
B: Adsorption methods  
C: Precipitation methods  
D: Homogeneous assays  
E: Turbidimetric, agglutination, etc., assays  
From: Gosling (1990)

assays, particularly those designed to operate in the picomole range or lower, involve a distinct separation step or even a series of such steps.

In BIA systems, the separation step serves the dual function of determining the proportion of enzyme label distributed between the free and antibody-bound fractions, and separating exogenous enzyme from sample components that might interfere with its measurement. The latter may include endogenous enzymes with similar substrate specificity, enzyme inhibitors or activators, and competing substrates. Generally, measurement of activity of the antibody-bound enzyme label is

preferred. A failure to do so has in many cases led to matrix interferences and background problems with low dilution samples.

The separation of the free form varies with the type of assay. In a competitive assay, separation removes the unbound enzyme tracer and free analyte so that only the antibody-bound fraction remains. If a low affinity antibody is used in the assay, this step may occasionally also remove the bound fraction to a varying degree. In an immunometric or sandwich assay, the separation is responsible for removing the unbound labeled antibody. In both assay designs, the efficiency of the separation has direct effects on the quality of the assay (Wild and Davies 1994). In competitive assays, the efficiency is reduced by the following:

1. A failure to remove all of the free enzyme tracer. The residual unbound tracer causes high background signal.
2. Partial removal of enzyme tracer bound to antibody, especially when low affinity antibodies are used in the assay.
3. Interference in the reaction between antibody and analyte, and
4. Inconsistent separation due to variability in the sample matrix.

In immunometric assays, the major source of error in practice is failure to remove all of the unbound labeled antibody.

To consider a separation technique for a routine immunoassay, five potential characteristics must be considered: speed, simplicity, applicability, reproducibility, and cost. In order to maximize assay precision and sensitivity, an ideal separation technique must ensure complete separation of the free and bound fractions with relatively simple and foolproof manipulations. The technique should not modulate the primary ligand-antibody reaction. It should also be accomplished rapidly, preferably without elaborate or expensive equipment. Furthermore, an ideal separation method should be unaffected by the constituents of the sample (whole blood, plasma, serum, urine, saliva, cerebrospinal fluid, feces, milk, etc.), be generally applicable to a wide variety of analytes, and be amenable to automation.

The "efficiency" of a separation technique can be defined as the completeness with which the bound and free forms are separated. In theory, a perfect separation system should completely divide the two components of the assay (i.e., the 100% efficient). However, in practice, this is seldom achieved. An incomplete separation often causes "misclassification errors," thereby resulting in assay bias and imprecision. Conventionally, the efficiency of separation can be measured as "nonspecific binding" (NSB) of the enzyme label to the assay tube. This is so-called diluent or assay blank.

NSB may occur due to physical trapping of the free ligand in the bound complex, the presence of impurities in the enzyme label with properties similar to those of the bound complex, adsorption of the free ligand to the assay tube, or

simply a failure to remove all the supernatant liquid (Newman and Price 1991; Chard 1978; Morris 1985).

NSB of the enzyme tracer is in fact the most common problem encountered in competitive EIAs. Such binding adds a level of background noise that can reduce precision, particularly at high concentrations. It can be minimized by using solid-phase or second antibody separation systems (described later in this section). In contrast, the separation in immunometric sandwich assays has a direct effect on assay sensitivity, i.e., the precision at very low and zero concentrations of the analyte. The separation in such assay designs can be greatly improved by repeatedly washing off the unbound, residual labeled antibody from the solid phase with wash solution.

## Classification

A wide variety of separation techniques have been used in immunoassays. All such techniques exploit the physicochemical differences between the ligand in its free and bound forms. The pioneering radioligand assays of the early 1960s utilized several different separation methods. The chromatoelectrophoresis method used by Yalow and Berson (1960) in their first RIA took advantage of differences between free and antibody-bound insulin in two properties: adsorption to cellulose and charge. Electrophoretic separation was also used by Ekins (1960) in his thyroid-binding globulin (TBG)-based thyroxine assay, and by Linner and Greenwood (1962) in their first growth hormone RIA. The double antibody methods were first developed by Hales and Randle (1962) and by Morgan and Lazarow (1962) for the insulin assay, and by Unger and his coworkers (1962) for the growth hormone assay.

Since these early developments, a great variety of separation methods have been developed. This reflects the fact that immunoassays are so universally applicable that no single separation method fits the properties of the multiple combinations of ligand-antibody (or receptors) that can be used as a universal basis for an assay. Furthermore, the performance demanded of a method depends to an extent on the purpose of the procedure. For example, an assay may be optimized for speed and ease of performance, or sensitivity and reproducibility. These requirements in turn determine the choice of separation technique.

Several methods are available for separating the free and bound forms (Table 7.1) and these essentially represent a chronicle of assay development and sophistication in the immunodiagnoses field. Among the groups listed in Table 7.1, all but the first group have achieved widespread use in immunodiagnoses. This is primarily because there is no single ideal separation method applicable to all systems. The first four methods described in Table 7.1 are liquid-phase (sometimes also referred to as solution-phase) systems where separations occur in solution. The walls of the assay tube are not actively involved in these reactions and there

TABLE 7.1. Classification of Separation Systems

Principle	Examples	Advantages	Limitations
Physicochemical properties of antibody or ligand	Chromatoelectrophoresis (starch gel, cellulose acetate, polyacrylamide gel), gel filtration (column or batch)	Generally applicable, well researched	Slow, equipment intensive, requires high level skills, complex steps, very low throughput
Chemical/fractional precipitation	Ammonium or sodium sulfate <sup>a</sup> , trichloroacetic acid, ethanol <sup>b</sup> , dioxane, polyethylene glycol (PEG) <sup>a</sup> , cross-linked dextran <sup>a</sup>	Inexpensive, fast, generally applicable, well researched	Multistep procedure, test performance suffers due to number of steps, messy reagents, requires a degree of laboratory skills
Adsorption	Coated charcoal <sup>c</sup> , silicates, hydroxyapatite	Inexpensive, fast, generally applicable, well researched	Multistep procedure, test performance suffers because of a number of steps, messy reagents, requires a degree of laboratory skill
Immunology	Double or second antibody precipitation <sup>d</sup> (applicable to both soluble liquid and solid-phase systems), protein A	Specific, fast, generally applicable, well researched	Multistep, requires centrifugation
Solid phase	Coated particles, membranes, discs, magnetic particles, tubes, microtiter plates, paddles, etc.	Easy, eliminates steps, improves performance, requires fewer skills, easier to automate, many product configurations, some eliminate handling and allow faster kinetics because of increased protein binding	Higher cost per test, kinetics are generally slow, large sample volumes

<sup>a</sup>At concentrations of  $\geq 200$  g/L

<sup>b</sup>Usually at 70% concentration at  $-20^{\circ}\text{C}$ .

<sup>c</sup>Binds free fraction.

<sup>d</sup>With or without PEG.

are no antibody-coated particles. They are sometimes used in competitive immunoassays.

Many of the parameters of a suitable separation technique are shared by both ELAs and their analogous RIA procedures. However, only the last two groups, viz., immunological precipitation and solid-phase separation, have found significant applications in ELAs, and of these two, solid-phase separation is the most commonly employed in commercial ELA products.

The principles of various separation techniques are briefly described below.

**1. Separations Based on Physicochemical Properties of Antibody and/or Ligand CHROMATOELLECTROPHORESIS.** Paper chromatoelectrophoresis was the first method described for separation of the reactants in a RIA (Yalow and Berson 1960). It evolved directly from the techniques originally used for the identification of antibodies to insulin in patients' serum.

For the purposes of separation of bound and free hormone, the technique depends on the fact that free hormone is adsorbed to the paper at the site of application, while the antibody-bound fraction migrates towards the center of the strip. After drying, sections of the strip can be counted to determine the distribution of radioactivity between the two phases.

Electrophoresis, however, has many practical disadvantages including high NSB (> 20%), and is rarely used in modern commercial products. It is also expensive and time consuming for routine use.

**GEL FILTRATION CHROMATOGRAPHY.** Because the ligand-antibody complex is much larger than the free ligand, they can be separated by gel filtration chromatography using a cross-linked gel matrix in the form of small particles, such as Sephad  $x^e$  or Biogel<sup>®</sup>. Two approaches have been used. In the first, the gel matrix is used in the form of a column. This procedure is much too complex and time consuming for routine application. In the second, more practical, approach, the gel is actually incorporated in the incubation medium. Low molecular weight ligand can then distribute freely both inside and outside the gel, whereas the ligand-antibody complex cannot enter the gel, thus effecting the separation of the two forms. Separation by batch addition of a gel or similar matrix was widely used in earlier commercial kits for the measurement of thyroid hormones.

Similar to electrophoretic methods, separation by gel filtration chromatography is expensive, time consuming for routine use, and gives high NSB.

**2. Chemical/Fractional Precipitation.** The above two techniques were soon superseded by fractional precipitation approaches, which were widely used in the 1970s and even the early 1980s. The general mechanism of fractional precipitation depends on the use of salts and solvents that reduce the amount of "free" water in a system. Immunoglobulins, which have the lowest solubility of all proteins, are precipitated in solvents and solutes such as ethanol, dioxane, and poly-

ethylene glycol (PEG), or are salted out by ammonium or sodium sulfate. Antibodies are almost completely precipitated in 33% saturated ammonium sulfate, in 70% ethanol, and in 15% PEG.

When the primary antibody-ligand reaction is completed, the separation material is added at a concentration in which the antibody and the antibody-ligand complex are insoluble and therefore precipitate, while the free fraction remains in solution. The precipitate is pelleted by centrifugation. The tracer activity is then determined in the pellet (bound fraction) or the supernatant (free fraction).

Precipitation methods have the advantage of being simple, rapid, and inexpensive, and usually do not require any special incubation period. They are also amenable to automated pipetting systems. Furthermore, they are highly reproducible and virtually devoid of lot-to-lot variation. However, NSB still tends to be high, in the range of 10-20%. These methods, however, are not suitable for ELAs. Ethanol precipitation methods have been used in assays for insulin and hCG; ammonium sulfate for assays of angiotensin, vasopressin, cyclic AMP, steroids, and prostaglandins; and PEG in a variety of immunoassays including growth hormones, thyroid hormone, and steroids.

**3. Adsorption Methods.** The nonspecific adsorption of biological molecules to particle surfaces is widely used as a method for the separation of bound and free ligand. Most such procedures depend on the fact that only the ligand and not the antibody or antibody-ligand complex have this property. Materials with a high adsorptive power include celluloses, glass powder, silica powder, talc, active charcoal, and fuller's earth.

Of the adsorption methods, the activated charcoal technique, first described by Herbert et al. (1965) for the vitamin B<sub>12</sub> assay, is the most extensively used. It may be used coated or uncoated. The coating, particularly with dextran, has been assumed to work like a molecular sieve, permitting only the small molecules from coming into contact with the adsorptive surface. However, a number of proteins including albumin, gelatin, and even serum may work equally well to reduce the binding of the antibody or antibody-ligand complex to the charcoal.

Typically, after the primary ligand-antibody reaction is completed, the adsorptive material is added and the tubes are centrifuged. These separation methods are unusual in that the free fraction is pelleted and the bound fraction stays in solution. The supernatant is decanted into another tube for counting.

Charcoal adsorption techniques have been extensively used in assays of small molecules, such as steroids, drugs, and peptides up to a molecular weight of approximately 10,000. Time and temperature are critical factors during the separation and centrifugation, and great care needs to be taken to ensure that a consistent amount of charcoal is added to each tube. Charcoal may also disturb the equilibrium of the antibody-ligand reaction by stripping off the ligand bound to antibody. For the same reason, charcoal should be left in contact with the reaction mixture for the minimum possible time.

4. *Double or Second Antibody Methods.* The introduction of secondary anti-species antisera was a major advance in introducing specificity for the separation mechanism of free from bound form in the immunoassay reaction. First introduced by Uhiger et al (1962) and Morgan and Lazarow (1962), precipitation of the bound complex with secondary antibody directed against the primary antibody is widely used as a separation procedure in RIA systems. The second or double antibody method is rapidly becoming the method of choice for an even wider range of assays, from those for small molecules to enzyme-labeled immunoassays.

Precipitation reactions occur only at high concentrations of ligand and antibody. The aim of this separation method is to create a sufficiently large immunological complex or micelle by incorporating the first antibody-specific antigen complex, so that it is possible to separate the bound phase by ordinary centrifugation. In this method, the first or primary antibody becomes the antigen of a second antigen-antibody reaction. Separation by this technique requires a relatively large amount of secondary antibody and, therefore, a correspondingly large amount of the species of IgGs of which the first antibody forms a part must be included. Thus, a second antibody system always involves addition of carrier protein, either whole serum or IgGs from the species in which the first antibody is raised.

To use this separation technique, it is necessary to test not only the concentration of antibody but also the appropriate concentration of carrier IgGs, because the optimal amounts will vary with each antiserum tested. The following factors thus need to be considered.

1. Completeness of precipitation of the bound complex. In the presence of an excess of the first antibody, this should represent nearly 100% of the immunoreactive tracer.
2. The minimum quantity of the "second" antibody required to achieve complete precipitation. Excessive amounts are likely to be both expensive and to lead to the problem of the "prozone" phenomenon, i.e., in the presence of an excess of antibody, immunoprecipitation may not occur.
3. The "assay blank," or the amount of tracer precipitated by second antibody in the absence of primary antibody. Ideally, this should be less than 5%. A high value can occasionally be due to the presence in the antiserum of antibodies directed to the ligand.
4. A second antibody system should be evaluated in the presence of the test sample (e.g., urine, plasma, serum, etc.) for which the assay is designed. A procedure that appears satisfactory in the presence of different buffers may nevertheless, in the presence of biological material, be subject to striking nonspecific effects. This is often reflected in precipitation of the bound complex or an increase in the assay blank, or both.

The double antibody separation can either be performed as a postprecipitation method, which is the most frequently used variant, or the precipitation may be

done before the primary reaction (preprecipitation). The latter works if the antibody-binding sites of the primary antibody are not sterically hindered after the precipitate has developed. Otherwise, it will prevent the primary antibody-ligand reaction. This modification can actually be regarded as a solid phase coupled antibody system where the immunoglobulin precipitate constitutes the solid phase. However, this method does not work with all double antibody precipitating antisera, and may have a tendency to decrease the sensitivity of the assay.

An alternative in which the double antibody is coupled to a solid phase (DASP) avoids some of the variability of the precipitation reaction. Coupling of the second antibody to an insoluble matrix such as cellulose (den Hollander and Schuur 1971) yields a system that is convenient and that does not require the use of carrier immunoglobulins. However, the precipitation and evaluation is time consuming, and therefore, the method is not widely used except in the form of commercially available reagents.

The main advantage of second antibody separations is the very low NSB (~2%). However, this method requires the need for a second incubation (1 hr to overnight, depending on the secondary antisera) that can more than double the overall time it takes to perform the assay. This problem is sometimes overcome by the use of accelerators of the precipitate formation, such as ammonium sulfate or PEG. PEG-assisted secondary antibody precipitation in fact combines the benefits of the second antibody and PEG methods, i.e., specificity and speed of the assay are enhanced. When used at low concentrations of 4%, PEG hastens the precipitation of the cross-linked matrix. Taking performance and convenience into account, this is probably the best of all liquid-phase separation methods.

5. *Surface-Coated Solid-Phase Systems.* The liquid- or solution-phase separation systems described above suffer from several disadvantages, including time-consuming preparations, longer assay times, high NSB, and the need for careful washing of the immunoprecipitates and refrigerated centrifuges. In order to overcome these difficulties and to provide a means of automating the separation technique, the solid-phase group of separation methods was developed. At present, they are the most popular separation methods used in EIAs.

In solid-phase assays, the antibody or the receptor is coupled to a non-soluble phase, either noncovalently by simple physical adsorption or by covalent coupling. This coupling is done in advance so that the antibody remains insoluble from the beginning of the assay. Antibodies coupled to such a solid phase or matrix are often referred to as "immunoadsorbents" or "immunosorbents." Because the antibody is now no longer freely in solution, it becomes much easier to separate it from the supernatant after the primary incubation step. The separation of the free from bound form can be achieved by centrifugation or filtration for particulate solid phases such as agarose, polyacrylamide, or polystyrene beads. Magnetic fields can be used to separate particles that have an iron core. The larger, dis-



pensable forms of solid phases such as tubes, cuvettes, microtiter plates, balls, dipsticks, and adsorbent devices allow easier and efficient separation of the two phases through simple rinse and decantation steps.

In the case of polymer-coated magnetic iron particles ("magnetizable" particles), separation involves placing the assay tubes after the primary incubation in a holder over a strong permanent magnet and decanting the supernatant. The unmobilized antibody-bound fraction is retained at the bottom of the tube.

The Seron MAlaclone™ assays use such magnetizable particles. These are liquid-phase immunometric assays in which the capture antibody is conjugated to fluorescein isothiocyanate (FITC). Following the first incubation, which occurs in solution, the genetic solid phase (magnetizable particles that have an attached antibody to FITC) is added and, following a second incubation, the particles are sedimented in a magnetic field. This design uniquely combines the fast kinetics of a liquid-phase assay with the low background signal of a solid-phase assay.

The first solid-phase system introduced was the coated tube method of Catt and Tregear (1967). Since then, an enormous range of solid-phase supports have become available for performing immunoassays. These range from particles of dextran and cellulose, continuous surfaces such as polystyrene or polypropylene tubes and microtiter plates, latex beads, and various membranes to more recently polymer-coated magnetic iron particles. Although they were first introduced in the mid-1960s, solid-phase supports gained popularity only in the 1980s. The relatively slow introduction of these techniques was primarily due to difficulties in producing reliable coating/coupling procedures to link the antibodies to the solid phase. This was particularly a major problem with tube and plate supports, and as a result, particulate solid phases were the first to receive widespread acceptance as their large surface area enabled more reproducible reaction conditions (Donini and Donini 1969; Bolton and Hunter 1973; Axon et al. 1967; Chapman et al. 1983).

The binding of antibodies to a solid phase may change its ligand binding characteristics. Therefore, immunosorbent antibody must be tested after coupling of the antibody. Both changes in avidity and significant loss of antibody quantity (sites) are known to occur as effects of coupling to solid materials.

Some solid-phase systems also offer distinct advantages with regard to the coating process, which requires considerable investment of time and money. It is therefore preferable not to have to do this for each individual antiserum. Moreover, this also results in over 90% wastage of the precious antibody. Thus, universal solid systems coated with substances such as streptavidin or protein A can be used for adsorption of the primary antibody. The former reagent can be used in conjunction with biotinylated primary antibodies.

Irrespective of the nature of the solid support being used, the use of solid-phase secondary antibodies offers several advantages over their first antibody counterparts. Such systems also provide a universal reagent for use in a wide range of as-

says provided the primary antibody is raised in the same animal species. This method also allows efficient use of the primary antibodies and does not affect the kinetics (the primary antigen-antibody reaction as sometimes happens when the primary antibody is attached to the solid phase. Moreover, being an excess reagent, the secondary antibody does not require precise dispensing, unlike a solid-phase primary antibody, whose concentration in the assay system is critical. Antigen-coated solid phases are also used in heterogeneous ELAs. Antigen-coated solid surfaces are often used in limited reagent systems and are, therefore, most appropriate to the measurement of small molecular weight analytes. However, the recent development of anti-idiotypic antibodies has now enabled the development of excess-reagent systems for such analytes (Barnard and Kohn 1990).

Solid phases as separation systems offer several advantages. Some of these include the following:

1. They can be applied to virtually any binding protein capable of physical or covalent attachment to solid surface.
2. They are highly efficient and produce virtually complete separation of the bound fraction.
3. NSB is very low (< 1%).
4. They give excellent precision if carefully tested.
5. They are not as liable as solution-phase separation systems to nonspecific effects introduced by plasma and serum.
6. The stability of the immunosorbent is considerably enhanced. For example, latex particle coupled antibodies are stable for over a year when stored as a liquid reagent (Thakker et al. 1991). Similarly, if solid phases are dried and stored desiccated, the antibody is stable almost indefinitely (Voller et al. 1979).

In spite of their several obvious advantages over solution-phase separation systems, solid-phase systems do suffer from certain drawbacks, which at least partially explains why these sophisticated systems are not in universal use. These include the following:

1. The preparation of primary reagent is tedious.
2. The recovery of antibody activity on the solid phase is only 30% or less of that in the original IgG preparation. This is probably due to the fact that many of the molecules attach to the solid phase through their binding sites. Such wastage is tolerable only if the supply of the antiserum is abundant.
3. In the case of antibodies developed for larger molecules, their attachment to solid phase often results in a loss of affinity, and hence, sensitivity in the assay. However, this factor is only critical in assays where extreme sensitivity is required.
4. The coupling process, whether covalent or noncovalent, sometimes results in the partial denaturation of the antibody, thereby changing its affinity constant. This

TABLE 7.2. Relative Merits of Solid-Phase Separation Techniques

Solid phase	Advantages	Limitations
Beads	Quantity can vary to suit assay, good surface areas, material can vary to suit assay, surface coating of other materials (magnetic), generally applicable, can be automated	Requires separations, multiple steps, large assay volumes
Tubes	Eliminates centrifugation, convenient, allows automation, generally applicable	Large assay volumes, expensive, limited selection of material, slow kinetics
Plates	Small reagent volumes, eliminates centrifugation, convenient, generally applicable	Waste for unused wells, requires special readers, slow kinetics, limited selection of material
Paddles	Flexible format, faster kinetics, larger selection of materials	Higher cost per test, difficult to automate
Membranes	Can be fabricated to suit assay, high binding capacity, faster kinetics, flow-through capability, dot binding format	Potentially more expensive solid phase

tends to increase the usage of precious primary antibody. However, the availability of monoclonal antibodies now renders this a financial rather than a volume consideration.

5. Immobilized antibodies tend to "leak" off during storage and during the assay. This results in loss of precision in the assay.
6. The actual assay procedure may become more complex and the washing of the solid phase to separate the free and bound fractions may require several washing and aspiration steps. This is particularly critical with assay designs that utilize particulate solid phase support systems.

The relative merits and weaknesses of different solid-phase separation techniques are summarized in Table 7.2.

## Errors of Separation

A confounding problem with solid-phase separation technologies is choosing between high antibody binding capacity and low NSB. Generally, these two parameters exhibit an inverse relationship. All solid materials have a tendency to adsorb small amounts of the label. In EIAs, because of the signal amplification effect, even very small quantities adsorbed will influence the assay results.

The degree of NSB is affected by the type of diluent, the concentration of the enzyme label used, the volume of the solid-phase material, pH, ionic strength, and the presence of other reagents. The inclusion of buffer salts, chaotropic reagents, proteins, and detergents in the wash solution is well known in most immunoassay development programs.

The physical separation of the bound and free forms is still one of the main sources of assay imprecision in heterogeneous EIAs. When the soluble phase is decanted from the solid phase, a small portion of the fluid may still be left in the tube after decanting. If it constitutes a substantial fraction of the soluble phase, variations in its volume can cause considerable assay variation. This type of error is particularly prominent if the total volume of the assay is small.

Repeated washing of the solid phase to separate the two phases often improves the assay precision, primarily by lowering the NSB of the enzyme label. The physical flushing that introducing a wash buffer produces is only one component of this effect. Repeated washings remove entrapped label, reduce adsorption of label to surfaces, and aid in the removal of the reaction supernatant.

The manner in which the wash buffer is applied depends upon the solid phase used. Particles, beads, microtiter plates, and tubes can be washed actively, whereas membranes will be washed by capillary flow or radial partition. When the wash flow rate is slow, as in the latter case, the use of detergents and proteins in wash fluid becomes even more important in reducing the NSB (Newman and Price 1991).

Washing and separation steps have been automated in a variety of systems. Some examples include microtiter plates for which various commercial plate washers are available, membranes (Abbott DMax<sup>®</sup>, OPIUS-PB<sup>®</sup>), magnetic particles (Serono-Baker<sup>™</sup> SR1<sup>®</sup>, TOSOH ALA<sup>®</sup>-600, and the Technicon Immuno-1<sup>®</sup>), and coated tubes (Boehringer Mannheim ES-600<sup>®</sup> and the Becton Dickinson Affinity<sup>®</sup> systems). The automation of these important steps of heterogeneous EIA systems constitutes one of the most important developments in automating immunoassays.

Finally, regardless of theoretical arguments, it should never be assumed that any separation technique is perfect and produces total separation of the free and bound fractions. Also, it should not be assumed that different separation techniques differ only in their efficiency. The fact that two different procedures yield identical results for assay blank and zero standard does not necessarily mean that the composition of these fractions is identical. Similarly, the results obtained with any separation procedure are almost certain to vary with the actual medium used in the assay (e.g., serum, plasma, urine, milk, etc.). Comparison of standards prepared in a diluent buffer with unknowns will generally reveal nonidentity. This is in essence an artifact of the separation technique itself. The only solution to this problem is to ensure that standards or calibrators are prepared in media as nearly as possible identical to that of the sample.

In the following section, important characteristics of the various solid-phase systems commonly used in EIAs are briefly described.

## SOLID-PHASE SYSTEMS

### Introduction

The solid phase in which the antibodies or antigens are immobilized is one of the most important elements in heterogeneous EIAs. It contributes to the reproducibility, accuracy, precision, and sensitivity of the EIA (Kennedy and Challacombe 1988; Ngo 1991). The simple manipulations that are generally required to separate the free from bound form immobilized covalently or noncovalently are probably the most important reason for the rapid increase in popularity of solid-phase EIA systems.

A wide range of solid phases are commonly used in immunodiagnostic products (Figure 7.2). They are also used in about 70% of all new assays currently being developed (Figure 7.1). In some solid-phase assays, the use of first (De Boever et al. 1983) or second antibody (Zaidi et al. 1988) conjugated to microcellulose particles or "microbeads" acts largely to accelerate precipitation of the immune complexes. However, mainly because they require use of a centrifuge, the use of microbeads has fallen from about 40% of new solid-phase assays in the early and mid-1980s to 10% or lower today (Figure 7.2). Magnetizable particles, which also offer large surface-to-volume ratios, have had continual popularity, being found in about 10% of new assays (Gosling 1990). Antibody-coated tubes, being used in about 15% of new solid-phase assays in the early part of the 1980s, but their use has declined with the more widespread use of 96-well microtiter plates and strips of similar wells. The growth in the use of microtiter wells has been steady, increasing from about 15% of new assays in 1980-1982 to about 70% in 1989. Precision-molded plastic balls and plastic sticks are used in about 5% of solid-phase assays. Antibodies immobilized on membranes are now an important factor, particularly in immunoassays formulated for over-the-counter sale or patients' bedside use.

According to Tijssen (1985), an ideal solid phase should have the following desirable characteristics:

1. High capacity for binding immunoreagents, i.e., high surface area-to-volume ratio.
2. Possibility of immobilization of many different immunoreagents.
3. Minimal desorption or "leakage" of the immunosorbent.
4. Negligible denaturation of immobilized molecule, and
5. Orientation of immobilized antibody with binding sites towards the solution and the Fe to the solid phase.

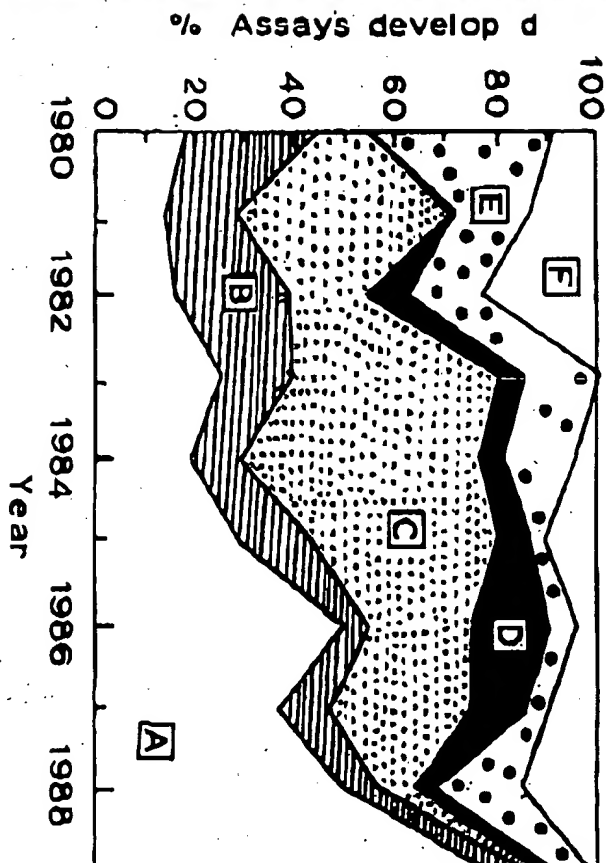


Figure 7.2. Trends over the past decade with respect to the use of different solid-phase separation methods. The general criteria for the inclusion of data were the same as for Figure 7.1. The microtiter wells were in plates (a total of 71 assays over the decade) or in strips (13 assays), with the latter increasing in relative popularity since they first appeared in 1983. The microbeads included microcrystalline cellulose, Sepharose® 4B, etc., coated with primary antibody or second antibody. The other solid phases included auto-exchange columns (1), inerted (2), capillary tube (2), indium slide (1), and "in situ generated solid phase" (1).

A: Microtiter wells  
B: Tube  
C: Microbeads  
D: Magnetizable beads  
E: Other solid phases  
F: Antibody-coated tubes  
From: Gosling (1990)

Solid-phase matrices commonly employed in heterogeneous EIAs are listed in Table 7.3. Polystyrene and plastics in general are by far the most popular solid phases, because their use makes the coating procedures extremely simple. Membranes, especially nitrocellulose, are replacing plastic in several EIA applications. Particulate solid phases are also very efficient because they may be dispersed throughout the reaction mixture and have a much higher ratio of surface area-to-volume.

The important characteristics of various solid-phase systems are described below.



TABLE 7.1. Types of Solid-Phase Supports Used in EIA

Type	Protein capacity	Method of binding
<b>Plastic Plates and Tubes</b>		
Polystyrene (PS)	300 $\mu\text{g}/\text{cm}^2$	Hydrophobic
Polyvinyl chloride	300 $\mu\text{g}/\text{cm}^2$	Hydrophobic
Unactivated microliter plates	100 $\mu\text{g}/\text{cm}^2$	Covalent, hydrophobic, hydrophilic
<b>Beads and Microparticles</b>		
PS beads	300 $\mu\text{g}/\text{cm}^2$	Hydrophobic
Derivatized PS beads	300 $\mu\text{g}/\text{cm}^2$	Covalent, hydrophobic, hydrophilic
<b>Microparticles<sup>a</sup></b>		
Protein A beads <sup>b</sup>	10 $\text{mg}/\text{ml}$	Covalent, hydrophobic
Activated beads <sup>c</sup>	20 $\text{mg}/\text{ml}$	Noncovalent
Membranes	10 $\text{mg}/\text{ml}$	Covalent
Nitrocellulose	100 $\mu\text{g}/\text{cm}^2$	Hydrophobic
PVDF <sup>d</sup>	100 $\mu\text{g}/\text{cm}^2$	Hydrophobic
Nylon	100 $\mu\text{g}/\text{cm}^2$	Hydrophobic
Modified membranes	100 $\mu\text{g}/\text{cm}^2$	Covalent, hydrophobic
Paper		
Derivatized paper	> 10 $\text{mg}/\text{cm}^2$	Covalent

<sup>a</sup> Less than 1  $\mu\text{m}$  in diameter and remain in colloidal suspension throughout the assay.

<sup>b</sup> For adsorption of antibodies only through the region.

<sup>c</sup> Include agarose, cross-linked agarose, carboxy agarose/polyacrylamide, and polyacrylamide.

<sup>d</sup> Polyvinylidene difluoride.

## Plastics

Plastic was one of the first solid-phase systems used in immunoassays. The separation of free from bound form on plastic is achieved simply by decanting, aspirating, or retrieving the solution. This simplicity has a direct positive effect on the reproducibility and sensitivity of the assay. Because they can be molded into many shapes, plastics are a convenient solid support for many immunoassays. Polystyrene, polypropylene, and polyvinyl chloride (PVC) are the most common plastics used in the diagnostics industry. Because PVC can be cut easily, it is an excellent matrix for RIAs in which quantitation is done by counting individual wells. However, because it is not translucent, EIAs that will be quantitated by a plate reader should be performed in polystyrene and polypropylene and not in PVC plates. A general description of the chemistry and manufacturing aspects of these and other types of plastics commercially available can be found in an annual publication, *Modern Plastics Encyclopedia* (McGraw-Hill, New York).

The forms of plastic supports for EIAs are varied. Some of these include tubes, microtiter plates, beads, discs, plastic-coated dipsticks, matchsticks, paddles, and even cocktail stirrers. Many other sizes and shapes can also be used as a solid phase plastic support in EIA systems. The commonly used forms are described below.

1. *Tubes.* Plastic tubes were first introduced by Cat and Tregear (1967). Tubes often give the best choice of assay surface. They offer flexibility in number of assays and in work volume, combined with acceptable handling possibilities and equally good use in ELISA and IRMA/RIA. Commercially, several competitive and immunometric EIAs have been developed that rely on antibody- or antigen-coated plastic tubes. They have also been automated by a number of manufacturers.

The choice of plastic is critical. Because it is easier to handle and is less breakable, polypropylene is generally preferred over polystyrene. However, there is little or no difference in the coating characteristics of these two plastics. The size of the tubes used in commercial EIA kits is typically 10–12  $\times$  70–75 mm. They may be conical, round, or flat bottomed; however, to provide increased surface area and better mixing during the assay, star tubes are generally preferred. These make the assays work better and faster. The surface-to-volume ratio can also be increased by rotating the tubes almost horizontally in circular test tube racks. Upon slow rotation, a relatively large surface is covered. Tubes generally require larger volumes than microtiter plates. Although 0.5–1-ml volumes are commonly used, up to 3–4 ml can be used.

Although they were one of the earliest solid-phase systems exploited, coated tubes are also one of the lowest capacity solid phases used in EIAs. Moreover, they have several other drawbacks. Although they are well suited for screening methods, tubes lack adequate precision for quantitative EIAs, primarily because of the variability in the amount of immunosorbent immobilized during the coating stage. Not only do tubes from different batches of the same manufacturer vary, but individual tubes of the same batch do so as well. The immobilized reagent also tends to "leak" off during storage and during the assay.

Uniform coating of the tubes with the immunoreagent is therefore absolutely critical to ensure precision in the final assay and to avoid very high rejection rates of batches of coated tubes. Especially for competitive assays, it is essential that all of the tubes are coated with an identical amount of antibody. This is generally achieved by carefully controlling all of the manufacturing conditions, including the processes used to manufacture these tubes.

Another major drawback associated with plastic tubes is the small surface area for antibody binding as compared to other solid-phase supports such as membranes and coated particles. In addition, the ligand-antibody reaction takes place only at the interface between the solid phase and liquid, thereby slowing down the

rate of the assay. However, assay kinetics can be greatly improved by agitation of the tubes during the incubation steps.

These drawbacks notwithstanding, the low binding capacity of coated plastic tubes can produce extremely low NSBs; values of less than 0.01% are not uncommon. Moreover, precoating and storing of tubes gives an optimal choice of protecting reaction surfaces provided airtight packs containing desiccant are used as packaging materials. Furthermore, the exact number of precoated tubes can be taken from storage without manipulating the reaction surfaces that are not going to be used.

Analogous to the use of coated tubes is the use of "dipslicks" and other such similar devices. Although not widely used, they are best suited for qualitative or semi-quantitative EIAs, and offer extremely simple assay protocols. Disposable p-hystyrene cuvettes are also used in some cases because they can be directly used with a common spectrophotometer. The volumes of such containers are quite large.

**2. Microtiter Plates.** Microtiter plates are probably the most popular solid phase in use at the present time. Their widespread application in the diagnostics industry required the development of automated plate washing systems as well as suitable plate-based detection systems. It was the delays in the development of these instruments that held back their introduction as long as it did (Newman and Price, 1991). A major reason for their popularity is that less labeling of the reagents is required, thereby reducing a very tedious and labor intensive process. Microtiter plates in general are not very well suited for RIAs. They are most commonly used with nonisotopic labeling systems such as enzyme, chemiluminescent, and fluorescence-based immunoassays.

Microtiter plates are available in a range of plastics treated in a variety of manners. Polystyrene and PVC are the most commonly used plastics for this purpose. Although PVC plates may adsorb antibodies more efficiently, they also tend to give high background levels and release little of the adsorbed immunoreactant. The most commonly used format is the 96-well ( $12 \times 8$  wells of 0.3 ml) microtiter plate form, where the wells provide convenient reaction chambers for immunoassays. However, strips as well as individual wells are also available commercially. The wells may have flat, U-shaped, or V-shaped bottoms. The choice between these shapes depends on the method used for quantitation of the EIA. With a read-through photometer, flat-bottomed wells give less variation, whereas for visual inspection, U-shaped wells are desirable (Tijssen 1985).

Microtiter plates suffer from similar drawbacks as those associated with coated plastic tubes. In addition, drift across the plate due to pipetting delays and temperature gradients across the plate due to their thermal insulating qualities are major problems. Assay kinetics can be greatly improved by incubating the plate at elevated temperatures (37°C) and using continuous vibration. In a conventional

air incubator, the wells at the corners warm up more quickly than ones in the center; thus, special plate incubators have been developed that provide consistent warming of all of the wells. Similarly, as their volume capacity is only about one-tenth that of the coated plastic tube, they also require strong signal generation systems to provide sufficient signals.

In spite of their theoretical limitations and difficulties associated with their manufacture, immunoassays based on microtiter plates as well as coated tubes have been very successful commercially. These assays provided early indications that the success of an assay system could be driven more by convenience than by superior technical performance. Because of their compact size, microtiter plates are the most convenient formats for manually performed immunoassays.

**3. Beads.** Until the advent of fully automated analyzers, bead assays were commonly used for immunometric sandwich assays. They are much larger than microparticles, so that just one bead is used for each test. The surface area offered by the bead is similar to that of a coated tube. They are also easier to manufacture than tubes and can be coated in bulk rather than individually.

## Membranes

Polymeric, microporous membranes are also used as a solid support for filtration-based immunoassays of a wide variety of analytes. They overcome many of the problems inherent in the solid phase immunoassays as they combine the qualities of a solid substrate with a range of expanded capabilities. The flow-through capability of membranes offers a distinct advantage to improve assay kinetics. Large fluid volumes can be actively moved past the membrane surface, improving the reaction kinetics between immobilized and soluble reactants. Thus, assay times can be significantly reduced to meet the demands of the market.

Microporous membranes are generally described as polymer membranes with a defined and measurable (rated) porosity in the range of 0.05–12  $\mu\text{m}$ . A microporous membrane is generally a thin-film membrane (100–200  $\mu\text{m}$  thick), composed of a polymeric lattice that is largely void volume (80% void volume). This provides a flat sheet material with a large surface area-to-volume ratio, which in the case of some polymer materials, binds proteins at a high density (Harvey 1991). Membranes are also available sealed to the bottom of wells in microtiter plates. With certain types of polymers, this large surface area can be chemically activated to provide a mechanism for covalent attachment of proteins.

Commercially, a wide variety of membranes is available, including nitrocellulose, cellulose acetate, regenerated cellulose, nylon, and polyvinylidene fluoride (PVDF). Glass fibers can also be used as a membrane support. The protein binding capacity differs with the type of polymeric material, with nitrocellulose and nylon providing the highest binding capacity. Nitrocellulose membranes also pro-

vide lower background signals than the nylon membranes. Cellulose acetate and regenerated cellulose membranes are generally insufficient to support a rapid immunoassay. The use of the hydrophobic PVDF membrane in immunoassays is generally limited, primarily because the membrane must first be wetted with a nonaqueous solvent. This characteristic renders hydrophobic PVDF membranes unusable for membrane-based immunoassay configurations.

Among the different types, nitrocellulose microporous membranes have found wide applications in qualitative and semiquantitative immunoassay systems. Nitrocellulose is prepared by the direct nitration of cellulose, usually to 10–15% nitrogen by weight. Nitrocellulose membranes have a high protein binding capacity of high affinity and offer a surface that is easily blocked. The binding of proteins to nitrocellulose is extremely fast, and therefore preparation of the membrane is very rapid. In addition, these membranes are available in a variety of different pore sizes, yielding different flow properties and protein binding capacities. The membranes also can be cast with fabric supports to significantly increase the tensile strength for subsequent device manufacture. Nitrocellulose membranes can be cast on porous plastic supports, creating a form of the material that plays a different role in some immunoassay systems. Such examples include lateral flow devices and dipstick configurations.

Although compatible with isotopic detection, the majority of membrane-based immunoassays have been developed for use with nonisotopic signals. Of these two types, ELA and particle capture agglutination assays have been most important. With the former, the use of substrates that yield insoluble colored products predominates, although enzyme-linked chemiluminescent assays have been developed more recently. Nitrocellulose membranes are particularly useful in assays where only the presence, and not the quantity, of an immunoreactant is to be established, when only very small amounts of samples (e.g., < 1  $\mu$ l) are available, and when ionic detergent-solubilized antigens are to be tested. Nitrocellulose membranes also offer great potential for providing a simple multianalyte approach by immobilizing several antibody "spots" on a single strip of membrane (Pappas 1988; Ekins and Chu 1991).

## Paper

As material purity is a primary consideration in the production of papers for analytical and diagnostic use, only pulps of the highest quality are used for their production. These are high  $\alpha$ -cellulose (that fraction of a pulp stock remaining undissolved after treatment of the pulp by 17.5% NaOH at 20°C for a total elapsed time of 45 min) pulps, which are obtained primarily from cotton linters and secondarily from sulfate-processed wood chips.

Diazotized paper is widely used for dot blot immunoassays in which the amount of protein bound to the sheet must be particularly high. The linkage to the

paper is covalent through free amino groups on the antigen or antibody. There are a number of commercially available sources for diazotized paper. The most stable of the derivatized papers is aminophenylthioether cellulose (APT paper). Diazotized paper is prepared by treating the APT paper with an acidic nitrite solution, converting it to diazophenylthioether (DPT) paper. The coupling is then done by adding the protein solution to the paper.

This most inexpensive form of solid phase has the advantage that large amounts of coated solid phase may be prepared. Paper also offers several additional advantages in the diagnostic devices as a support medium. The bulk of the applied test solution is rapidly wicked away from the point of application/site of reaction and the products that are formed there. This capillary/filtering action assists in the creation of sharply defined reaction zones and the removal of potentially obscuring soluble secondary reaction products and/or other colored materials contained in the originally applied sample solution. Furthermore, the smooth, white background of the paper itself provides an ideal contrast for any colored reaction products formed thereon. Paper thus provides a multiplicity of functional benefits as a reagent support and dispersing media and as a sample application, test component immobilizing, concentrating, and filter media.

## Glass

Antigens or antibodies can also be immobilized onto glass surfaces by heating, fixation with formaldehyde, or coupling with glutaraldehyde to aminocapillary glass surfaces. However, glass is not widely used in ELA systems.

## Particulate Solid Phases

Particulate solid phases have originally been used for the separation of radiolabeled ligand-antibody complex from free labeled antibody in immunometric assays. Since then, their applications have been extended to both hapten and protein assays and with all detection systems developed for heterogeneous ELAs.

Particulate solid phases for immunoassays offer several advantages. These include:

1. The speed of the assay compared with microtiter-based assay increases considerably. A comparison of particle-bound antibodies versus an equal amount of antibody on the wall of the microtiter wells demonstrates that the particles captured all the antigen in 5–10 min, whereas a microtiter well required 24 hr (Musted et al. 1991).
2. Batchwise preparation of solid-phase antibody allows easy monitoring and quality control.
3. They provide freedom to modify surface characteristics in order to reduce interference and allow antibody to be immobilized with a variety of methods.

4. There is no limitation on the amount of solid-phase antibody that can be used in an assay and no antibody variation between samples.
5. Magnetizable particles are applicable to rapid isolation of particulate antigens.
6. The combined use of defatted particle-size mixtures, each coated with its own uniquely specific capture antibody and flow cytometry, allows development of multianalyte assays. These also allow for the inclusion of an interference assay in the package.
7. There is no limitation on sample size.

Polymer particles also have some drawbacks. They are not readily adapted to automated washing procedures. Furthermore, they tend to sediment during the incubation period, which necessitates continuous shaking during the incubation step.

Particulate solid phases can be broadly divided into two types: nonmagnetic and magnetic. Their properties are briefly described below.

**1. Nonmagnetic Particles.** The nonmagnetic particulate solid supports include glass, latex, Sepharose<sup>®</sup>, Sephadex<sup>®</sup>, Sephacryl<sup>®</sup>, and nylon particles and beads. The choice of a particular support depends on the relative coupling capacities of the different plastics and the size and density of the particles.

The carbohydrate polymers, viz., Sephadex<sup>®</sup> and cellulose, have higher antibody binding capacities than polystyrene, nylon, or glass particles. However, the absolute binding capacity of the particles depends upon the surface area available, i.e., on the size of the individual particles.

Among the various nonmagnetic particulate solid phases, latex is the most common. Latex particles were first used by Singer and Plotz (1956) in an agglutination test for rheumatoid factor. Since then, they have been widely used for several agglutination tests in clinical diagnostics including pregnancy detection kits, in serodiagnostics for bacterial typing and identification, and for measuring serum blood levels of several antibiotics. In contrast, their use in EIAs has been fairly limited. Latex particles have a similar density to water, so they stay in suspension during the incubation. Hence, the kinetics of the assay are efficient, requiring shorter incubation times.

Most of the currently available latex particles are produced by emulsion polymerization. This process is best suited for producing submicron (< 1  $\mu\text{m}$ ) particles, but can be extended for particles up to 3  $\mu\text{m}$ . A suspension polymerization process is used to make particles of up to 10- $\mu\text{m}$  size. Very large particles (> 100  $\mu\text{m}$ ) are produced by the suspension process. However, it yields particles of a rather broad size distribution as compared to the emulsion process.

There are essentially two types of latex polymers: homopolymers, produced by the copolymerization of a single type of monomer and represented by polystyrene and polyvinyltoluene; and copolymers, produced by the polymerization of more

than one type of monomer, and represented by styrene-divinylbenzene, styrene-butadiene, vinyltoluene-*t*-butylstyrene, and styrene-vinylcarboxylic acid.

The four basic ingredients of the emulsion polymerization process are a monomer, water, an emulsifying (surface-active) agent such as potassium laurate, sodium dodecyl sulfate, or sodium ditherylsulfosuccinate, and a free-radical-initiating agent such as potassium persulfate or hydrogen peroxide. The polymerization process involves a particle-nucleus initiation phase and a subsequent particle growth phase.

Commercially available latex suspensions are primarily composed of polymer particles (30%) and water (> 69%), with small amounts of surfactants (0.1–0.5%) and inorganic salts (0.2%). Surfactants dissolved in the aqueous phase and adsorbed on the particles assist in the stabilization of the particles. Their complete removal from the suspension makes the latex particles less stable and more prone to flocculation. Latex particles have an inherent negative surface charge provided by covalently bound sulfate groups on their surfaces. Carboxylate-modified and amide-modified latex particles contain active carboxylic and carboxamide hydrophilic groups, respectively, on their surfaces. Latex particles produced in water look like milk or white paint, and may occasionally develop an iridescent or metallic sheen. Dyed latex particles are also available commercially.

Separation of particulate solid-phase systems is usually by centrifugation and decantation. In microparticle capture enzyme immunoassay (MBIA), the latex particles are captured by a filter consisting of a glass-fiber matrix. Particles may also be captured by membrane filtration. The advantage of MBIA and membrane-based systems is that the particles may be easily washed, thereby consistently reducing the background signal.

**2. Magnetizable Particles.** A natural progression from latex particles led to the inclusion of iron oxide in cellulose or latex particles to form paramagnetic microparticles. These are commonly used as a solid phase with the advantage of a separation that does not require centrifugation. Separation is achieved by the application of a magnetic field, which draws the particles to the side or base of the tube. The supernatant is then removed by aspiration or decantation (Wild and Davies 1994).

For use in EIAs, the magnetizable particles should fulfill the following requirements.

1. The incorporation by the matrix of high amounts of magnetic material.
2. The possibility of easily activating and subsequently coupling proteins to the magnetized matrix.
3. A relatively high mechanical stability to prevent the beads from fragmenting and thereby avoiding interference from the magnetic material in the assay.
4. Ease of manufacture, and
5. Homogeneity with regard to size and amount of incorporated magnetic material.

The use of magnetic supports for use in immunoassays dates back to the early 1970s. The advantages of a magnetizable particle are a high surface area, rapid analyte capture, and properties that lead to efficient separation and washing. Paramagnetic ferrous oxide-based magnetizable particles are the most commonly used in commercial applications. The ferrous oxide is incorporated into a cellulose matrix to which the antibody is coupled to provide a stable reagent with low NSB. This was later extended to develop a magnetizable charcoal reagent by co trapping charcoal and ferrous oxide in a polyacrylamide gel. This approach considerably enhanced the use of charcoal as a separation matrix for hapten assays. It not only eliminated the centrifugation step, but also minimized adverse effects on the primary ligand-antibody reaction (Al-Dujaili et al. 1979).

Ferrous oxide has thus far proved far superior to other magnetic components, primarily because of its small particle size (10-20 nm) and good magnetic response. The size is, however, dramatically increased during entrapment in the cellulose, the final particle size being determined by milling (1-3  $\mu$ m). Chromium dioxide has also been employed as a particulate solid phase as it has less residual magnetism than iron oxide, and hence, is easier to resuspend during the important washing process. Chromium dioxide also results in lower NSB.

Not all magnetic separation systems are based on suspensions of micro particles. The solid-phase ALA<sup>®</sup>-PACK range of assays manufactured by TOSOH consists of twelve 1.5-mm polymer beads coated with ferrite. An external magnetic field keeps the beads in motion during the incubation (Wild and Davies 1994).

The first magnetizable particle assays used either batch processing in magnetic racks (e.g., Corning), or automation in continuous flow systems. More recent automation has included unit dose systems such as the Sero-Baker<sup>™</sup> SR1<sup>®</sup>, and the TOSOH ALA<sup>®</sup>-600. Magnetizable particles have also been used in assays as secondary antibody separation systems and coupled to primary antibodies, for use in assays for haptens and proteins using a range of detection systems from isotopes, enzymes, and fluorophores to chemiluminescence (Newman and Price 1991).

## IMMOBILIZATION OF IMMUNOREAGENTS

### General Considerations

Immunoreagents, either antibodies or antigens, can be immobilized onto solid phases in one of three different ways.

1. Adsorption to predominantly hydrophobic surfaces
2. Covalent attachment to activated surface groups, and
3. Nucleoavalent, electrostatic and hydrophilic bonds between a molecule immobilized by either of the above two methods, which in turn is coupled to a ligand, e.g., an antibody-antigen or biotin-avidin bond.

The extent to which these methods are chemically discrete is not absolute (Butler 1991). In any event, the solid phase should not be considered a passive component in the process of adsorption or coupling of antibody or antigen. The physical coupling of an antibody restricts its movement. The degree to which this influences the reaction kinetics depends upon the nature of the solid-phase surface and on the surface area of coupled antibody in relation to the volume and concentration of the other immunoreagents.

Compared to solution-phase assays, the ligand-antibody reactions in solid-phase assays are much slower. In such assays, the forward rate constant  $k_f$  is reduced while the dissociation constant  $K_d$  is increased, thereby increasing the equilibrium association constant (Newman and Price 1991). However, because the binding reactions are generally irreversible, as generally is the case with high affinity antibodies, the surface ligand-antibody reaction can be considered multivalent. This significantly increases the chances of reassociation. The overall reaction rates, however, can become limited by the rate of diffusion of the solution-phase components as the surface concentrations become depleted.

A number of studies have been conducted, especially with polystyrene and membranes, to understand the processes involved in the immobilization of immunoreagents onto the solid phases. Indeed most solid phases used in the ELISAs are mildly to strongly hydrophobic, thus providing the potential for secondary hydrophobic interaction even when the primary interaction is covalent. In fact, studies on covalent immobilization of proteins to microparticles have shown that, although the initial binding is > 80% covalent, the effective bonds after incubation for 16 hr can be > 80% noncovalent (Butler 1991). Direct covalent coupling of immunoreagents to hydrophobic surfaces might therefore be considered a means of facilitating secondary hydrophobic interactions. The immobilization of solid-phase immunoreagents is thus chemically heterogeneous and varies with the specific surface, ligand chemistry, and conditions of immobilization.

Similar solid phases manufactured by different companies also tend to exhibit different behaviors regarding protein immobilization. Kenny and Dunsmoor (1983) evaluated 11 different polystyrene microtiter plates from four different companies and found essentially two types: one that adsorbs albumin poorly, and another that adsorbs it well. IgG was adsorbed well on both plate types. Plates that bind albumin well are best suited for mixtures of antigens; however, background signal tends to be higher. A 100-fold excess of a nonspecific protein during adsorption essentially prevents the detection of the antigen.

Plastic plates exhibit a significant variability, not only among the various lots, but also among the wells of the same plate. Irrespective of the origin of the plates, the coefficient of variation of absorbance can range from 5% for the wells of one plate to 30% of the other for the same lot. The "edge effect" (i.e., wells of the perimeter adsorbing more protein than those in the interior of the plate) is particularly notorious when one works with the microtiter plates (Cressman and Den-



mark 1978; Kricka et al. 1980). Because of this reason, as many as 40% of the available wells may not be used by the researchers.

The edge effect has been attributed to differences in surface characteristics of the plastic (Burt et al. 1979) and to thermal characteristics (molding temperature, cooling) being different from those in the interior (Denmark and Chlessum 1978). Thermal gradients generated during incubation may thus play a critical role in producing the edge effect. Polystyrene is a poor conductor of heat, and therefore, a thermal gradient may exist between the outer and inner wells during an incubation period of 30 min or longer, with initial and final temperatures of 20°C and 37°C, using a routine laboratory incubator. The use of a forced air incubator may eliminate this edge effect, giving a higher assay precision and reproducibility. Warming both the plate and the solution to the incubation temperature prior to the addition of the solution also seems a simple alternative (Tijssen 1985).

Furthermore, each protein also appears to have its own unique affinity for the hydrophobic polymer surface (Figure 7.3). Thus, within the linear binding region, the ratio of bound/free protein is constant, but the percentage adsorbed is protein specific (Butler 1991). A similar difference in affinity was observed by several researchers (Brash and Lyman 1969; Lee et al. 1974; Tijssen 1985). In this regard, high molecular weight biomolecules appear to adsorb best, probably because of multisite attachment to the solid phase.

Passive adsorption thus takes place predominantly by means of hydrophobic interactions between the solid-phase and the liquid-phase immunoreagents. In order to adsorb one molecule onto the solid phase, other molecules must be removed. If this process leads to a gain in entropy, a firm attachment can be achieved. The presence of polar groups in the solid phase also increases the binding capacity of water-soluble molecules such as antibodies (Rasmussen 1990). The binding strength in hydrophobic interaction is dependent on the number of binding sites (interactions) between the molecules and the solid phase. Larger molecules with potentially many binding sites, as mentioned earlier, will therefore adsorb better and with higher stability than smaller molecules with low affinity for hydrophobic interaction.

Although immobilization of antibodies and antigens on a plastic solid phase by hydrophobic interactions has proven to work extremely well in an increasing number of ELAs, the need for a more specific coupling as well as the need for coupling a wide range of molecules is quite obvious. For example, ELISAs of small peptides are sometimes difficult to perform because of the poor adherence by these peptide antigens to the plastic surfaces of commercially available, inexpensive ELISA plates. With small peptides, the hydrophobic interactions are frequently too weak to permit detection of the final antigen-antibody complex (Dagenais et al. 1994). In such cases, covalent attachment of the solid-phase immunoreagent may permit a higher concentration of reagent to be immobilized than by simple adsorption. Furthermore, a specific binding with potential for specific orientation

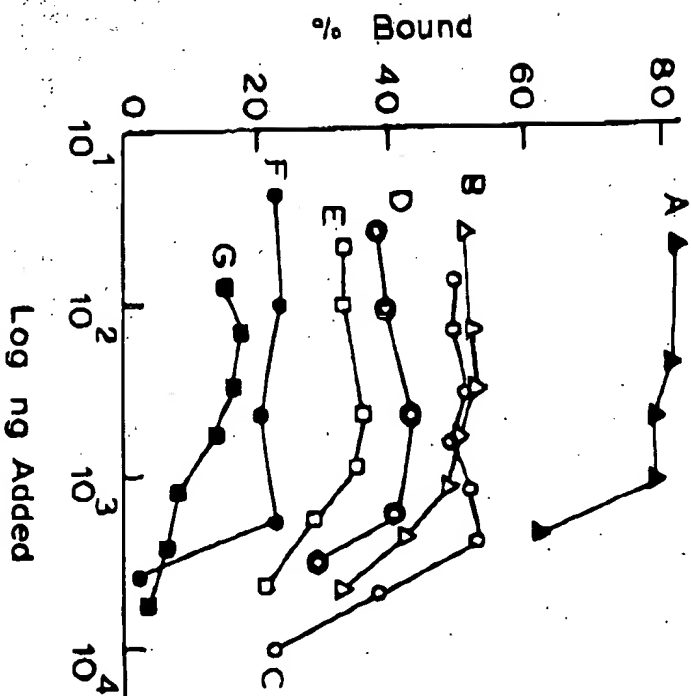


Figure 7.3. The adsorption characteristics of seven different proteins on polystyrene surface. Within the linear binding region, the ratio of bound/free protein is constant, but the percentage adsorbed is protein specific, because of each protein's own unique affinity for the hydrophobic polymer surface.

A: Bovine IgM

B: Bovine IgG1

C: Ovalbumin

D: Bovine IgG2

E: Bovine serum albumin

F:  $\alpha$ -Lactalbumin

G:  $\alpha$ -Lactalbumin

From: Cantacaro et al. (1980)

of the molecules can only be established by covalent coupling where only one binding site is needed to give a stable coupling with high binding strength.

\* Covalent coupling of immunoreagents to solid phases offers some advantages. These include the following:

1. Studies have shown that one can attach 10-40% more protein covalently than by adsorption (Douglas and Montalib 1994; Bangs and Mera 1995).
2. Covalent coupling may provide more precise control of the coating level when the desired protein coverage is low.
3. Covalent coupling binds protein more securely. This is beneficial in production of tests or assays that are so sensitive that they would be influenced by minute quantities of IgG that might leech off the solid phase over time.

4. The covalent bond is thermally more stable. For example, after 1 hr at 56°C, 99.7% of covalently linked IgG remained bound, compared with only 70% of adsorbed IgG (Bangs and Meza 1993). This property could be essential with solid phases, such as microparticles, that are to be used in polymerase chain reaction or other applications requiring thermocycling.
5. Covalent coupling conserves costly reagent because it does not require the large excess of protein necessary for the adsorption process. Because excess reagents are not used, the multiple layer phenomenon present with passive adsorption is also less of a problem.
6. Hydrophilic molecules must be covalently linked to solid phase. Unless bound to the surface, they will surely desorb when the equilibrium is disturbed by removal of unbound soluble molecules from solution. The smaller antibody fragments, such as Fab<sub>2</sub>, Fab, or Fv portions, do not normally adsorb well.
7. Covalent introduction of a spacer arm allows a secure but flexible attachment of many different molecules to the solid phase. Covalent attachment of hetero- or homo-bifunctional and trifunctional crosslinkers (available from Pierce Chemical Co., Rockford, IL) also facilitates coupling of ligands with unusual available chemical groups.
8. Directional binding (e.g., periodate oxidation of vicinal hydroxyls on the carbohydrate portion at the Fc portion of IgG and binding of the oxidized IgG to hydrazide-activated matrices) ensures that binding sites are directed outward to the solution and remain accessible. Thus, binding is no longer a random event.
9. Covalent attachment at relatively few sites may overcome the "Goulier effect," whereby large, well-adsorbing protein molecules become tightly adsorbed over so wide an area or at so many contact points that they become distorted or denatured.
10. With some solid phases, covalent coupling may be the only viable alternative. For example, monoclonal antibodies with pI around 4.0 cannot be adsorbed on latex particles, which tend to flocculate in suspension at this pI. Thus, covalent coupling of such monoclonal antibodies to latex particles may be easier than adsorbing them. Similarly, proteins do not adsorb well to hydrophilic silica microparticles; they must therefore be linked covalently to any of several coupling groups.
11. Covalent coupling also permits the use of high concentrations of surfactants (up to 1% Tween ) in the assay design to eliminate nonspecific binding. Such a high concentration of surfactant could easily dislodge the adsorbed but not the covalently coupled protein. Moreover, it reduces matrix interference.
12. Covalent coupling allows for "small peptides, nucleic acids, and other "non-adsorbable" molecules to be bound to a solid phase. Also, molecules that are present in too low a concentration to establish stable immobilization by adsorption may be firmly bound by covalent coupling.
14. Biomolecules that need detergent for solubilization cannot be adsorbed by passive methods. Thus, covalent coupling is preferred in such instances.

The concern over the stability of noncovalently adsorbed immunoreagents onto solid phases has therefore stimulated a great deal of research aimed at improving

the bond stability between the immobilized protein and the solid phase. I regard particular attention has been given to modify polystyrene surfaces! binding capacity and/or improved binding stability.

Several companies at present market a variety of solid phases containing reactive groups to covalently couple immunoreagents. For example, Nunc Inc., Denmark, has introduced microtiter plates that are coated with a secondary amine (methyl amine). Thus, any ligand carrying a carboxylic acid group can be linked covalently to the plate using a condensing reagent such as a water-soluble carbodiimide. Costar (Cambridge, MA) also markets covalent plates and strips that are prepared by modifying polystyrene surfaces by a post molding treatment to yield either a carboxylated or aminated surface. Biomedical Products Ltd., Rockaway, NJ, recently introduced microtiter plates with a hydrophilic surface of reactive aldehyde groups that enable the binding of amino group-containing ligands. Covalent immobilization of antibodies through its amino groups is generally not preferred, as these groups are involved in antigenic binding reactions. Non-amino acid groups such as carbohydrate chains on the proteins are often not involved in biological functions; thus, they can be used to covalently couple the antibodies onto the solid phase. BioProbe International (Tustin, CA) recently introduced microtiter plates (Avdublate-HZ™) with hydrophilic hydrazide surface groups capable of binding to the Fc portion of oxidized antibodies. Unlike other microtiter plates designed for covalent binding, no expensive and unstable cross-linking reagents are required for this purpose. Furthermore, any immobilized antibodies have their antigen-binding sites facing away from the solid phase, thus allowing maximal antigen binding.

Covalent coupling can also be performed with solid phases other than polystyrene microtiter plates. Surface-modified polymer microparticles are available commercially. These are often made by copolymerizing styrene with a small amount (< 5%) of a functional monomer, such as acrylic acid. This yields microparticles covered with carboxylic acid groups. Other monomers are used for this purpose with different surface chemistries. For example, native silanol groups on the surface of silica microparticles are readily reacted with aqueous or solvent-based silane coupling agents to yield preactivated silica particles with a large variety of surface functional groups. Examples include chloromethyl, carboxyl, and amino groups (Bangs and Meza 1993). Thus, oligonucleotides can be covalently bound to surface-modified silica via the 5'-amino end, while lipids can be bound via the  $\omega$ -carboxyl group on the fatty acid chain and propylamine surface groups on the silica (Haggen 1994).

Chemically modified surfaces often exhibit greater protein binding capacity. For example, bromoacetyl-functionalized polystyrene beads exhibit up to a 10-fold greater capacity for protein binding than unmodified polystyrene (Peterson et al. 1988). Moreover, no detectable dissociation occurs, unlike that observed with simple adsorption. The bromoacetyl coating is produced by chlorosulfon-

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## Product Development

## ENZYME IMMUNOASSAYS

### FROM CONCEPT TO PRODUCT DEVELOPMENT

This unique reference provides a pragmatic approach to the development of successful commercial immunodiagnostic products based on enzyme immunoassay technology. Presenting both basic and applied principles, **Enzyme Immunoassays** gathers information on all aspects of this process, from the initial conceptualization to the introduction of the product to the market.

Skillfully organized into two parts, this comprehensive resource begins by discussing basic concepts, such as the classification, structure and function of antibodies and the properties and characteristics of the most widely used enzymes in immunoassays. The second section examines the industrial product development process, including a detailed outline of various protocols and points to be considered for designing a successful product. Described in this section are the various formats available for product development, reagent formatting and assay development, data processing, standardization, scale up, and commercial manufacture of the product. Also included are the various regulatory requirements, the importance of good laboratory and manufacturing (GLP and GMP) practices, and international requirements such as the ISO 9000 certification process. Finally, information is presented for the benefit of entrepreneurs who would like to venture into this exciting field with their own company.

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